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THE EFFECT OF NIACIN AND ITS ANALOGS ON THE GROWTH AND ON THE NICOTINAMIDE COENZYME CONTENT OF *STAPHYLOCOCCUS AUREUS*

By

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The levels of nicotinamide coenzymes and the free adenine nucleotides were examined after growth in several media, with or without the addition of niacin and/or thiamine, in order to determine whether the levels of these nucleotides were related to the changes in glucose catabolic pathways in staphylococci that occurred following these vitamin additions. The proportion of glucose metabolized via the hexosemonphosphate (HMP) pathway by resting staphylococcal suspensions was stimulated 3-fold when the cellswere grown in a Vitamin-free Casitone growth medium to which niacin had been added in amounts $\geq$0.25 µg/ml. Concomitantly, there was a 10- to 15-fold increase in the NAD level, and a 2- to 3-fold increase in NADP. When 0.1 µg/ml of niacin was added, there was maximal growth but no stimulation of NAD synthesis or of HMP pathway activity. With the addition of higher niacin concentrations to resting niacin-deficient cells, metabolic changes similar to those in growing cells occurred. Even in the presence of protein inhibitors such as chloramphenicol, puromycin, or actinomycin D, the HMP pathway activity was increased, and the usual increases in NAD(P) were observed following the addition of niacin. Since the specific activities of the enzymes of the HMP pathway and of the other enzymes tested remained unchanged under conditions that precluded enzyme synthesis, any changes in enzyme levels observed in growing cells supplemented with niacin were secondary to those of the nicotinamide coenzymes, and to the stimulation of glucose oxidation via the HMP pathway. In order to determine if NAD was effecting glucose oxidation via the HMP pathway, staphylococcal suspensions were incubated for 6 hr in buffered (pH 7.0)-glucose, under conditions that allowed the NAD to decrease and the NADP to remain constant. After 2 hr, when the initial NAD concentration (2.2 µmole/g. dry wt.) had decreased to 1 µmole, the % of glucose oxidized via the HMP pathway dropped to 9%, reaching a value of 6% after 6 hr at which time the NAD had dropped to 0.5 µmole/g. The NADP concentration (0.45 µmole/g) did not decrease during the 6 hr period.

Upon readdition of niacin to the buffered-glucose, the NAD content of these cells increased to 1.2 μmole/g after 2 hr additional incubation of the cells, and the HMP activity increased to 16%. NAD, apparently by increasing NADP turnover via the transhydrogenase system, is controlling the activity of this expandable HMP activity.

Following growth in unsupplemented Vitamin-free Casitone growth medium, staphylococci had ATP/AMP ratios close to 1. With the addition of niacin and thiamine to the growth medium, which stimulated the HMP and TCA cycle activities, the ATP/AMP ratios increased to values >5.

In the presence of thiamine, 22 of the 1-, 2-, 3-, or 4-substituted pyridine compounds could completely replace the niacin requirement when they were added in either a synthetic medium, or in Vitamin-free Casitone; 3-substituted pyridine compounds such as 3-pyridylcarbinol and 3-acetylpyridine, were the most effective. Another 11 analogs yielded reduced rates of growth. There were 10 analogs, including 6-hydroxynicotinic acid, that were without effect, and 4, including 6-aminonicotinamide, that were inhibitory. Experiments using labelled quinolinic acid, isoniazid, and pyridine-N-oxide demonstrated that the analogs were converted to NAD(P) and not to the analogs of NAD(P). Other experiments with these labelled compounds showed that the requirement for high concentrations of analogs for growth was due to their limited uptake at low concentrations. Staphylococci were able to use many niacin analogs in concentrations greater than 1 mg/ml in the medium. Of the 40 compounds studied, precise structure to activity relationships were not evident, except that 3-substituted compounds were the most active.

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ABBREVIATIONS

AP	Acetylpyridine
ADPR	Adenosinediphosphoribose
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
APAD	3-Acetylpyridine adenine dinucleotide
6AN	6-Aminonicotinamide
6ANAD	6-Aminonicotinamide adenine dinucleotide
DPT	Diphosphothiamine
ED	Entner-Doudoroff (pathway)
EDTA	Ethylene dinitrilo tetraacetic acid, disodium salt
EM	Embden-Meyerhof (pathway)
G6P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
6PGD	6-Phosphogluconate dehydrogenase
GTP	Guanosine triphosphate
HK	Hexokinase
HMP	Hexosemonophosphate (pathway)
ICD	Isocitric dehydrogenase
LD	Lactic dehydrogenase
MK	Myokinase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide-reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-reduced
NDA	Nicotinyldiethylamide
NMN	Nicotinamide mononucleotide
NNO	Nicotinamide-N-oxide
PC	3-Pyridylcarbinol
PD	Pyruvate decarboxylase
PEP	Phosphoenolpyruvate
PK	Pyruvate kinase
6PG	6-Phosphogluconate
6PGD	6-Phosphogluconate dehydrogenase
PSA	Pyridine-3-sulfonic acid
TCA	Tricarboxylic acid (cycle)
TH	Transhydrogenase
Tris	Tris(hydroxymethyl) aminomethane
VFC	Vitamin-free Casitone

INTRODUCTION

Staphylococci are known to metabolize glucose aerobically and anaerobically by both the Embden-Meyerhof (EM) and the hexosemonophosphate (HMP) pathways, with the EM pathway predominating. The operation of the glycolytic pathway provides energy in the form of ATP, and the precursors for the biosynthesis of cellular components. The pyruvate produced by glycolysis is oxidized by way of the tricarboxylic acid (TCA) cycle.

In staphylococci, glucose oxidation through the HMP pathway is greatly stimulated when niacin is present in the growth medium. As for the maximal operation of the TCA cycle, staphylococci must be grown in the presence of both niacin and thiamine.

The requirement of niacin and thiamine as growth factors for staphylococci has been known for over 20 years. The only known function of niacin is to serve as the precursor for the coenzymes, NAD and NADP. The coenzymes are involved in the oxido-reductive reactions with the dehydrogenases. However, recent reports of NAD serving as a substrate for the DNA ligase, and for the regulation of cell division, point out some other important aspects of the NAD coenzyme.

In all living systems, NAD, NADP, and DPT are essential for the operation of the TCA cycle; NAD is important for the EM pathway, while the NADP and DPT are required for the operation of the HMP pathway. The operations of the HMP pathway and the

TCA cycle have been reported to be regulated by the availability of NADP and of DPT to these pathways in mammals and bacteria. On the contrary, increased concentrations of NADH in cells normally suppress the operation of these pathways by inhibiting certain enzymes in these pathways.

The effect of niacin and thiamine on the glycolytic pathways is relatively unexplored. It is known that these vitamins stimulate the overall operation of the glycolytic pathways and the TCA cycle. The purpose of the present study is to determine whether some of the factors, such as the relative concentrations of NAD(P), and the free adenine nucleotides, could influence the activities of the glycolytic pathways and the TCA cycle in staphylococci. Our data indicate that the relative operation of the HMP pathway and the TCA cycle activity is dependent on the total NAD(P) content of staphylococci. We found that many niacin analogs are able to replace niacin for the stimulation of growth, the operation of the HMP pathway, and the TCA cycle in staphylococci. Also, the NAD(P) content is increased in cells grown in the presence of the analogs.

HISTORICAL REVIEW

Staphylococci of medical significance are presently classified into two species: Staphylococcus aureus and Staphylococcus epidermidis. Most strains of S. aureus produce coagulase and phosphatase and are able to use mannitol anaerobically. Further divisions of this species into strains are based on antigenic analysis, biochemical characteristics, antibiotic susceptibility, and phage typing (29).

Staphylococci metabolize glucose predominantly by the EM pathway, and partly by the HMP pathway. Under anaerobic conditions, pyruvate produced from glucose is completely oxidized to CO_2, and water by the TCA cycle. Under anaerobic conditions, there is incomplete oxidation of glucose to pyruvate, glycerol, and lactate. Pyruvate can be further metabolized into lactate, acetate and CO_2.

Glucose repression of the TCA enzymes and pathway in staphylococci and other bacteria has been demonstrated by many investigators (15,17,19,29,33,42,123), and some of the terminal products of the glycolytic process have been shown to cause the repression. Gershanovitch et al. (33) observed that the accumulation of lactate in the cell repressed the synthesis of the TCA enzymes. From a detailed study of the levels of the TCA cycle enzymes in yeast grown on a different carbon source, Polakis et al. (100) proposed that the repression of the formation

of the TCA cycle enzymes was due to ATP itself, or to a closely related high energy intermediates. Magasanik (79) proposed the term 'catabolite repression" as a general phenomenon to describe the repression of the catabolic pathways. On the whole, the levels of the TCA enzyme biosynthesis are influenced by 1. the presence or absence of molecular O_2; 2. the repressive effect of glucose (catabolite repression); and 3. the balance between catabolite and anabolic demands on the cycle (37).

Besides glucose, the iron content in the medium has also been shown to have a marked effect on the glycolytic pathways and the activity of the TCA cycle. Theodore and Schade (120) noted that iron-rich cells yielded CO_2 from glucose-6-^{14}C in an amount equal to half that produced from glucose-6-^{14}C whereas iron-low cells produced no CO_2 from glucose-6-^{14}C. Iron is known to be necessary for the formation of heme proteins that are required for many enzyme-catalyzed biological oxido-reduction reactions.

Knight (63,64) first noted that niacin (nicotinic acid) and thiamine were both essential for the growth of staphylococci. In fact, this was the first report relating that niacin was a vitamin. Kligler et al. (61) later indicated that there was a relation between the presence of niacin and thiamine, and the utilization of glucose by Staphylococcus aureus. Staphylococcus increased the rate of pyruvate oxidation when both niacin and

thiamine were present in the growth medium. They concluded that niacin was involved in glycolysis while thiamine was needed for the aerobic breakdown of pyruvate. Recently Montiel and Blumenthal (84), and Idriss and Blumenthal (50), using resting cells of S. aureus incubated in the presence of labelled glucose-1-^{14}C and glucose-6-^{14}C, found that the addition of niacin to the growth medium increased the percentage of glucose metabolized via the HMP pathway while both niacin and thiamine were necessary for the maximal TCA cycle activity.

The pyrimidine and the thiazole moieties of thiamine added together were as effective as the intact thiamine molecule for the growth of staphylococci. Thiamine, in the form of diphosphothiamine (DPT) is recognized to serve in oxidative and non-oxidative decarboxylations which involve the TCA cycle, The decarboxylations involve the conversion of pyruvic acid to acetyl CoA in association with coenzyme A and lipoic acid, and the conversion of α-ketoglutarate to succinylCoA. In the HMP patthway, DPT involvement in the transketolase activity is well documented. For instance, Sauberlich (105), and Brin et al. (12) noted that the addition of thiamine to thiamine-deficient red blood cells stimulated the HMP pathway. In mammalian tissues, the main action of transketolase appears to be related to ribose-5-phosphate metabolism (105).

Witt and Heilmeyer (129) reported that the DPT coenzyme

could be an inducer of the pyruvate decarboxylase (PD) apoprotein. They observed that the PD activity increased by 50% during incubation of the yeast cells with glucose and thiamine, and by 300% when the cells were incubated with glucose, thiamine, and a nitrogen source. The reason for the higher activity of PD after incubation of the yeast cells was due to a stimulation of the de novo synthesis of the enzymes and not to an activation of the pre-existing enzyme molecules. Witt and Neufang (130) recently found that the DPT-dependent transketolase was also induced by thiamine. Greengard and Gordon (38) also believe that the cofactor of a mammalian enzyme, in addition to regulating the activity of the existing enzyme, may also influence in vivo the amount of the protein moiety of the same enzyme.

Das and Chatterjee (22) found that pyrithiamine, an analog of thiamine, could cause a partial block of the HMP pathway and a slight stimulation of the TCA cycle of a "pyrithiamine-adapted" (actually a mutant) strain of S. aureus. In another study, however, Telegdy-Kovats (119) found that oxythiamine stimulated the synthesis of thiamine in lactobacilli.

The only known function of niacin, or niacinamide, in microorganisms is to serve as a precursor of NAD and NADP, which both play a central role in cellular metabolism. The conversion of NAD to NADP is mediated by an ATP-dependent NAD kinase.

Iandolo et al. (49) noted that the growth of S. aureus

MF 31 was suppressed when grown in association with lactic streptococci. This inhibition was found to be due primarily to the nutrient depletion of a vitamin. The major factor necessary to reverse this inhibition was nicotinamide. Nicotinamide was found to increase the incorporation of ^{14}C-formate and 2-^{14}C-glycine into adenine when administered to mice (110). Both mammalian and bacterial NADase are known to be inhibited by nicotinamide. The addition of nicotinamide to liver tissue decreased its endogenous respiration by competing with NAD (31).

There are various strains of Pseudomonas fluorescens which are able to grow on synthetic medium in which niacin is used as a carbon source (69). Under anaerobic condition, Pseudomonas species could reversibilly oxidize niacin to 6-hydroxynicotinic acid and further to propionic acid, acetic acid, CO_2, and ammonia (48). Kligler and Grossowicz (60) observed that niacin was not an essential growth factor for Salmonella paratyphi A, but was essential for the fermentation of carbohydrate by this organism and that it must first be converted to NAD.

Davis (23) has designated the pathways that serve both anabolic and catabolic function as amphibolic pathways. These pathways encompass the enzymes of glycolysis, such as the EM and HMP pathways, and the TCA cycle. The amphibolic pathways provide carbon skeletons for biosynthetic purposes, the generation of energy in the form of ATP, and the hydrogen for the

electron transport chain. However, these pathways are under strict regulatory control either by enzyme repression or by end product inhibition (feedback inhibition). Reduced pyridine nucleotide (NADH) has been shown to play a major role in the regulation of these amphibolic pathways (41,104).

In the HMP pathway, both glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) achieve oxidation of their substrates by coupling with the reduction of NADP to NADPH. The HMP pathway has been proven to be rate-limited by the availability of the NADP sypply to the NADP-linked G6PD in a variety of mammalian tissues (13,111,112,126), and microorganisms (17,28,83,118). Also, the TCA cycle has been shown to be rate-limited by the supply of NADP to the NADP-linked isocitric dehydrogenase (ICD) (27.28). Eagon (28) noted that the HMP pathway and the TCA cyle activities were directly regulated by the NADP supply to the NADP-linked G6PD and ICD respectively. Microorganisms that have both the NADPH oxidase and transhydrogenase are found to utilize predominantly the HMP and/or ED pathways, both of which involve NADP as a coenzyme. However, some microorganisms could circumscribe the availability of NADP supply by acquiring modified enzyme systems such as having NADP oxidase or transhydrogenase and G6PD, and 6PGD that could use either NAD or NADP as cofactors. For instance, in Leuconostoc species (24), the G6PD can use both NAD and NADP

as cofactors. Recently, Barker (7) reported that NADP and NADPH could also serve as inducers of G6PD synthesis. Other investigators have noted that the HMP pathway could be rate-limited by the amount of G6PD (96,109). Osmond and Ap Rees (96) demonstrated that the HMP pathway in yeast was controlled by the amounts of G6PD present in the cells. The inhibition of NADP-linked G6PD by ATP and GTP seemed to indicate that the overall regulation of the HMP pathway activity involves more than one factor (6,75,78,107).

The majority of the studies on the nicotinamide coenzymes have been concerned with their relative coenzyme concentrations in cells and the ratios of the oxidized and reduced forms of the coenzymes. The NAD level has been found to be present always in greater concentrations than that of NADP level, whereas NADPH level is present in greater concentrations than that of NADP level (16,34). London and Knight (77) were able to classify bacteria into three groups based on their intracellular NAD content: high NAD (>4.5 μmole/g. dry wt.) in obligate anaerobes, medium NAD (1.0-3.0 μmole/g. dry wt.) in facultative anaerobes and photosynthetic bacteria, and low NAD (<0.9 μmole/g. dry wt.) in obligate aerobes. However, no difference of NADP content could be observed between the three groups. The intracellular NAD content was also partly dependent on the growth medium and the physiological conditions of growth. They

also found with *E. coli*, which does not require niacin as a vitamin, that the addition of 1.5 μg/ml of niacin to a minimal medium led to an increase of NAD from 2.4 to 9.4 μmole/g. dry wt. and to a lesser increase in the NADP level. In a different study, Iizuka and Mizuno (51) observed that only those bacteria which preferentially used nicotinamide had a NAD turnover, and that the degradation product of NAD was exclusively nicotinamide. There was no NADP turnover in the bacteria tested.

The regulation of cellular metabolism by adenine nucleotides is well documented (4,5,23,41,104,132). Since the enzyme concentrations in the cells are remarkably constant (35), it is most probable that the operation of any pathway may be regulated by the existing nucleotide levels. It has been suggested that the relative concentration of ATP and other nucleotides, even at physiological levels, could control key enzyme reactions that channel carbohydrate through the glycolytic and oxidative pathways. Yeast isocitrate dehydrogenase had been shown to be activated by AMP but not by ADP or ATP (5). The activation of isocitrate dehydrogenase by AMP could serve as a feedback mechanism to increase the intracellular levels of NADH and subsequently by ATP. Berry (11) demonstrated that the relative concentration of the nucleotides in the liver tissues played a major role in regulating the rate of pyruvate oxidation and carboxylation, both of which were stimulated when the ATP/ADP

ratio was maintained at greater than 6. Krebs (71) postulated that it was the ATP/ADP ratio in a cell, rather than the absolute value of ATP, or ATP/ADP ratio, that was controlling the large number of enzymes which are involved in carbohydrate metabolism.

Tsuboi et al. (121), and Tsuboi (122) found in mammalian erythrocytes, which lack a functional TCA cycle, that the glycolytic capacity was directly dependent on the ATP supply and not the NAD content. On the other hand, the TCA cycle and enzymes are known to be affected by the reduced nicotinamide coenzyme, NADH. NADH has been shown to be a powerful allosteric inhibitor of many bacterial enzymes and this inhibition could be completely overcome by low concentrations of AMP. NADH inhibitions of the citrate synthase has been demonstrated by Weitzman and Jones (125) in all Gram-negative bacteria, but not in Gram-positive bacteria such as staphylococci. They found that the citrate synthase activity of E. coli, in contrast to that from mammals and yeast, was regulated by the NADH concentration. Within the cell, this kind of inhibition may be regarded as a feedback control of end product of the dehydrogenating reactions of the TCA cycle. Furthermore, it is believed that the NADH concentration, as well as the adenylate system, may both play an important role in regulating the entry of pyruvate and acetylCoA into the TCA cycle in E. coli (5).

NAD, besides functioning as a cofactor in the dehydrogenase systems, is also involved in allosteric regulation of enzymes and cell division at the genetic level. Morton (85) proposed that the concentration of NAD in the cell is the regulator of the rate of cell division and he found a correlation between the nicotinic mononucleotide adenytransferase activity and mitosis. Zimmerman et al. (133), and Olivera and Lehman (94) found that the bacterial DNA ligase utilized NAD as a cofactor for the formation of phosphodiester bond in a non-oxidative manner. This reaction required a 5'-phosphoryl terminus on the DNA strand and the presence of the 3'-terminus of a suitably juxtaposed hydrogen-bonded DNA strand in the formation of a 3'-5' phosphodiester bond. In the process, NAD is cleaved to 5'-AMP and nicotinamide mononucleotide (NMN).

We found (46) the only major change which occurred after the addition of niacin to staphylococci was the marked increase of NAD and the doubling of NADP and this was correlated to the tripling of the oxidation of glucose via the HMP pathway. However, it is conceivable that the doubling of NADP, although dwarfed by the 10- to 15-fold increase in NAD, may be enough to cause the stimulation of the HMP pathway. Takebe and Kitahara (118) reported higher NADP content in the lactic heterofermentors as compared to homofermentors, and they attributed to the elevated HMP pathway to the higher NADP content.

It is known that different species of bacteria require different nutrients for niacin synthesis. Alternate pathways for niacin synthesis are available in certain bacteria. Under aerobic conditions, yeast could utilize tryptophan for the synthesis of niacin and under anaerobic conditions, another pathway would be operative (2). In E. coli, the precursors of niacin are a 4-carbon dicarboxylic acid, and either glycerol, or a compound to which glycerol can be easily metabolized (95). Xanthosomonas pruni (106) and Neurospora (97) could use the tryptophan-quinolinic acid pathway for NAD synthesis. In mycobacteria, quinolinic acid was considered as the intermediate formed by the condensation of a three carbon compound with aspartic acid (59). Both aspartic acid and asparagine stimulate niacin production in this organsim. So far none of these pathways have been reported in staphylococci. Organsims, which cannot use glycerol and aspartate or tryptophan for NAD biosynthesis, normally use the Preiss-Handler pathway, and they require niacin for growth. In E. coli and mycobacteria, quinolinic acid is probably the precursor for NAD biosynthesis, and niacin is a by product. However, the de novo pathway of niacin synthesis of niacin non-requiring bacteria is inhibited when niacin is included in the growth medium.

Coenzymes, although required in catalytic amounts, are normally produced in excess (127). However, little information

is available about the control of the coenzyme production. Imsande and Pardee (53) observed that the biosynthesis of nicotinamide coenzymes in E. coli was controlled by a repression mechanism that seemed to regulate the biosynthesis of nicotinamide mononucleotide pyrophosphorylase, the first enzyme of the NAD pathway.

The breakdown of NAD is effected through the action of NAD glycohydrolase (NADase) or pyrophosphatase. The NADase hydrolyzes the nicotinamide-ribose linkage, resulting in the formation of adenosinediphosphoribose (ADPR), and nicotinamide as products whereas NAD pyrophosphatase hydrolyzes the pyrophosphate bond between adenine nucleotide and nicotinamide mononucleotide. Nicotinamide specifically inhibits the NADase and this provides a simple way of distinguishing between the two processes of NAD breakdown. The NAD pyrophosphatase in Staphylococcus aureus exhibited no activity until after a heat-labile inhibitor was destroyed by boiling for 1-2 min (117). The association of a heat-labile inhibitor with NADase has also been reported in mycobacteria and Proteus species. It is interesting to note that the Proteus species grown in nutrient containing yeast extract possess no heat-labile inhibitor associated with the NADase as compared to those cells that have heat-labile inhibitor when they are grown in minimal medium. The NADase activity could be inhibited by nicotinamide in the

organisms. NADase could regulate the rate of glycolysis by controlling the amount of NAD in animal tissues since there was an inverse relationship between the NAD content and NADase activity. Tumor tissues also had a lower NAD content because of a very high NADase activity (81).

Anderson et al. (3), and Kaplan et al. (57) had demonstrated that a large number of pyridine compounds which were substituted in the 3-position could undergo exchange reactions in vitro with the nicotinamide moiety of NAD in the presence of certain animal NADase to form the corresponding coenzyme analogs. These compounds included isonicotinamide, pyridine, ethylnicotinamide, isonicotinic acid hydrazide, and acetylpyridine. In some cases, coenzyme analogs such as acetylpyridine coenzyme (APAD), can function in most NAD dependent dehydrogenase reactions. Deamido-NAD was essentially as active as NAD in a system in which the pyridine nucleotide hydrogen transferring enzyme came from Pseudomonas fluorescens and several animal sources. Deamido-NAD was less than 50% as active as NAD when it reacted with yeast alcohol dehydrogenase. NAD analogs other than 3-acetyl-pyridine are usually less active and tend to be more inhibitory in the dehydrogenase reactions. NAD analogs which lack a carbonyl group in position 3 were found to be devoid of coenzyme function. The introduction of a methyl group into the 4 or 5 position, or an amino substitution into the 5 position, of

the nicotinamide ring of NAD causes loss of coenzyme activity. The formation of APAD in tissue homogenate and whole animal has been observed and this formation could be inhibited by nicotinamide. Injection of 3-acetylpyridine into mice led to a rise in the total pyridine nucleotides in most tissues; NAD, but not the APAD, was found in the liver, whereas in the brain, spleen, and tumors, APAD was found (56). So far, only AP and 6AN have been reported to be incorporated into the coenzyme forms when they were administered to mice (25,54,55). Interestingly, 6AN has been used as an antagonist of the vitamin niacin in attempts to use it for the chemotherapy of certain virus diseases and of cancers. Johnson and McColl (54,55) detected 6AN coenzyme (6ANAD) in the liver and kidney of mice that were treated with 6AN, and in the neoplastic tissues from rats. The analog coenzyme was inactive with the yeast alcohol dehydrogenase and did not form an addition compound with cyanide. The tumor bearing rats were observed to withstand the lethal effects of 6AN better than the non-tumor bearing controls. The Walker adenocarcinoma tissue showed a marked lowering of ATP, ADP, and increases in AMP concentration, when 6AN was administered. The enzymes, 3-phosphoglyceraldehyde dehydrogenase and α-ketoglutarate oxidase were also affected (67). Both AP and 6AN are known to cause toxic effects on the central nervous system and niacin deficiency in dogs. It is gemerally believed that the toxicity

of these niacin analogs is caused bythe formation of abnormal NAD(P) analogs and the reduction in the normal intracellular NAD(P) levels. Furthermore, 6ANADP is known to be a competitive inhibitor for several NADP dependent enzymes (44). So far, there is no report on the formation of NAD analogs in microorganisms although many niacin analogs are reported (8,40,82,108) to be inhibitory. In one instance, it has been shown that the depletion of NAD from the cells was the primary effect of the niacin analog, isoniazid, was to cause a decrease in NAD due to the indirect activation of NAD glycohydrolase (10,114,128). Thus, a decrease in the concentration of NAD caused by the presence of the analog appeared to interfere with the normal function of the cells.

Preliminary investigation in our laboratory (124) indicated that many niacin analogs, given at higher concentrations, were able to replace niacin as a growth factor. These analogs also stimulate the percentage of glucose oxidized by the operation of the HMP pathway. Earlier studies by other workers on the effect of the niacin analogs on growth were controversial and the reports seemed to indicate that very few niacin analogs could replace niacin as a growth factor. Pyridinesulfonic acid has been reported to inhibit the growth of S. aureus (82), by acting as an antimetabolite for niacin. On the other hhand, Pitsch and Nakamura (99) observed that certain bacterial species

could use pyridinesulfonic acid in place of niacin as a growth factor. However, maximal growth stimulation of many niacin analogs occurred at a concentration of approximately of 1 mg/ml whereas only 10 to 100 µg/ml of niacin yielded maximal cell growth.

Hughes (47) observed that 5-fluoronicotinic acid could act as a potent inhibitor for staphylococcal growth and coenzyme synthesis. He postulated that the 5-fluoronicotinic acid was converted to 5-fluoronicotinamide, which then inhibited the NAD synthetisizing enzymes. This inhibition was reversed competitively by niacin, nicotinamide, and NAD. Streightoff (116) found that of the 10 analogs that had substitution at the 5-position, only 3 showed 50%, or more inhibition. 5-Fluoronicotinic acid was most inhibitory against streptococcal species, E. coli, and Lactobacillus plantarum, but failed to inhibit the growth of S. aureus. He suggested no reason as to why an analog could inhibit one species of bacteria and not the other. Landy (74) reported that trigonelline, pyridine betaine β-carboxylic acid, α-amino pyridine β-carboxylic acid, were completely inactive as growth factors for S. aureus. Only nicotinylglycine exhibited growth promoting activity when given in the same order of concentration as niacin. Knight (64) found that quinolinic acid, picolinic acid, isonicotinic acid, trigonelline, nicotinic diethylamide, nicotine, pyridine β-sulphonic acid, β-picolinic acid, and 2,4-dimethylpyridine could not replace niacin as a

growth factor. Pelczar and Porter (98) found picolinic acid, quinolinic acid, pyridine betaine carboxylic acid, and trigonelline, when added to the growth medium in 2 X 10^{-5} M concentration, could not support the growth of bacteria. We found that the concentration of niacin analogs that the previous workers used for growing bacteria were relatively low, somewhere in the range of 1 X 10^{-4} M, and this could attribute to their failure in observing the stimulation of growth by these analogs. A dose response on the growth by vitamins has been shown with niacin and nicotinamide. Koser and Kasai (70) noted that somewhat larger amounts of nicotinamide (3 X 10^{-4} M) were needed to support growth whereas no growth occurred at the ordinary vitamin level of 1 X 10^{-5} M. On the other hand, excessive amounts of niacin, or niacinamide (ca. 10^{-2} M) inhibited growth in a synthetic medium.

In general, it is assumed that compounds showing growth stimulation are those which are readily converted to nicotinic acid, or nicotinamide. Earlier surveys of active compounds seem to indicate that esterification of the carboxyl group of niacin results in a decreasing activity with increasing length of the alkyl chain. Substitution on the amide nitrogen of nicotinamide usually resulted in the loss of activity. Compounds active with microorganisms have all been shown to be utilized by higher organisms, and the microorganisms are considered to

be more specific for the niacin analogs. An excellent summary of all information relating to vitamins and microbes can be found in the recent book by Koser (68).

The exact mechanism by which niacin analogs can substitute for niacin is not known. One possibility is that the analog is converted to niacin, which is subsequently incorporated into the formation of coenzymes. This has been observed with the administration of labelled acetylpyridine, resulting in the formation of labelled NAD (9). Another possibility is that the analogs are directly incorporated to form coenzyme analogs, which can function in the place of the normal coenzyme. This possibility is unlikely since most of the NAD analogs formed are either inactive or are actually inhibitory to the dehydrogenase systems. It is also conceivable that the analog induces a change in the biosynthetic pattern of the microorganism, modifying them in such a way that the organism then begins niacin synthesis. This phenomenon had been observed in yeast in which the presence of the thiamine analog, oxythiamine, resulted in the increased synthesis and accumulation of thiamine in the cells.

The N-oxides of pyridine compounds have been reported to be a group of highly reactive compounds (93). The biological reduction of the various N-oxides are known but there is little information about the enzymes that could reduce the nicotinamide

oxide to nicotinamide. May (80) demonstrated the reduction of pyridine-N-oxide to pyridine by fermenting yeast. In this system, picoline-N-oxide could not be reduced. Niacin requiring strain of Lactobacillus arabinosus, and E. coli could both use nicotinic acid-N-oxide instead of niacin for growth but with reduced efficiency (86). Nicotinamide-N-oxide has long been known to be a common excretory product of niacin and niacinamide metabolism in mammals (68).

Pyridine has an aromatic ring structure having resonance stability of the same order as benzene but ring substitution of pyridine is more difficult than benzene. The 3-, or β-position of pyridine has typical aromatic properties, but the 2-, 4- and 6-positions have anomalous behaviour. The electron attraction by the ring N reduces the electron density in the 2, 4, and 6 positions. However, in niacin, the situation is enhanced by the presence of the carboxyl group. Both the N atom the carboxyl group increase the reactivity of the 2,4, and 6 position with regard to negatively charged hydroxyl ions (32).

At present there is no satisfactory explanation as to why niacin analogs were required in much higher concentrations as compared to niacin in order to stimulate the growth of niacin requiring microorganisms. Since the pattern of the intermediary metabolism appears to be fundamentally similar in all cells, the characteristic difference in the uptake of the niacin

analogs by the cells could then depend largely on the properties of their permeases. The actual mechanism for the specific permeation system is a very complex process and the permeation systems is depended on the permeability barrier, the stereo-specific permease portion, and the dependence of the permeation on the formation of a reversible permease-substrate complex. The utilization of many organic compounds (carbohydrate, organic acid, amino acid) into the bacteria had been shown to be controlled by specific permeases (18,76). The permeation system in turn is regulated by repression, feedback inhibition (26), induction, and catabolite repression (26,39,102).

Kawaski et al. (58) reported that the uptake of ^{14}C-thiamine by E. coli involved an active transport and that this was depended upon the temperature, pH, and the availability of a readily available energy supply. This uptake system involves a specific "carrier" protein which probably binds with the free thiamine to enter into the inner face of the cell membrane. At the inner face of the membrane, it is thought that the free thiamine "carrier" complex dissociates and releases the free thiamine which can then react with thiamine kinase and ATP to produce diphosphothiamine (DPT). In fact, the thiamine-deficient lactobacilli were able to take up thiamine in amounts at 200 times greater than their intracellular concentrations, and this uptake was considered an enzyme

mediated reaction (89). Rose (103) showed that niacin uptake by yeast was associated with a requirement for biotin. In the rat and human erythrocytes, the uptake of niacin was mediated by the process of diffusion and enzyme catalyzed conversion to nucleotides (73).

Since nicotinamide coenzyme analog formations from niacin analogs have been reported in mammalian tissues, it is interesting to investigate whether the same thing occurs in bacteria. The exchange reaction that involved the coenzymes appears to be a rapid means of producing coenzyme analogs in vivo and consequently could be used as a rapid means for inhibiting or altering cellular metabolism. It is also possible that the process of derepression by niacin could result in the synthesis of nicotinamide coenzymes. For instance, derepression of thiamine biosynthesis by adenosine resulted in the synthesis of a large amounts of thiamine in the washed-cell suspensions of Salmonella typhi LT_2 (90,91). For our investigation, the staphylococcal cells will be grown or incubated in phosphate buffer with labelled niacin analogs, such as tritiated pyridine-N-oxide, ^{14}C-quinolinic acid, and ^{14}C-isoniazid, to determine whether the pyridine compounds themselves are being incorporated into NAD and NADP. Staphylococcal cells will also be incubated with labelled succinic acid and glycerol to determine whether they are being incorporated for the formation of pyridine

nucleotides. Studies will also be made to determine whether the stimulation of growth and glycolytic pathways is actually due to the formation of normal coenzymes, or NAD analogs that have the same oxidative-reductive potential as the normal coenzymes. Comparative studies between niacin and the niacin analogs uptake will be determined to find out any differences in their degree of uptake.

MATERIALS AND METHODS

A. Methods

a. Bacterial cultures. The various strains of S. aureus, and S. epidermidis used in this research were obtained from the following sources: S. aureus Towler strain from Dr. W. C. Noble, St. Mary's Hospital Medical School, London, England; S. aureus 18Z from Dr. F. Kapral, Ohio State University, Ohio; S. aureus 2, 10 from Dr. S. Cloutier, University of Montreal, Canada; S. aureus serotype 1, 3, 4, the S. epidermidis strain ATCC 115, 12228, and 14990 were obtained from Dr. W. W. Yotis of this department; and Shigella flexneri, Proteus morgani, and Proteus vulgaris were from the department culture collection. Most experiments were performed with the Towler strain of S. aureus unless mentioned otherwise. The stock cultures were maintained on a 2% Trypticase-soy agar slant (Baltimore Biological Laboratories, Baltimore, Md.) at 4 C and were transferred to fresh slants every two weeks. Inoculated slants were incubated at 37 C for 24 hr and then refrigerated. Prior to the inoculation of the starter culture medium, a loopful of cells were taken from the slant and streaked onto a Trypticase-soy agar plate and incubated overnight at 37 C. A loopful of the freshly grown cells from the plate were then used to inoculate the starter flask. It was necessary to go through this procedure each time in order to obtain growth uniformly in the presence

of the 2% Vitamin-free Casitone starter culture medium within 6 to 8 hr.

b. Preparation of liquid media. Vitamin-free Casitone (Difco Laboratories, Detroit, Mich.) medium was prepared in a concentration of 2% (W/V) in distilled water. This medium is an enzymatic hydrolysate of casein that has been extracted with alcohol to reduce the vitamin content of the medium to very low levels. However, it is not completely "vitamin-free". Analysis provided by Difco Laboratories allowed us to calculate that a 2% solution of the Vitamin-free Casitone contained <0.00013 µg/ml of thiamine and <0.0132 µg/ml of niacin. The cells were grown either in 200 ml Erlenmeyer flasks containing 100 ml medium or in 2500 ml Erlenmeyer flasks containing 1000 ml medium. All flasks were carefully cleaned with 2% (V/V) FL-70 Biogradable detergent (Fisher Scientific, Pittsburg Pa.), and rinsed first with tap water and then with distilled water. The medium was sterilized by autoclaving for 15 min at 121 C. Sterile vitamin solutions were prepared separately by Millipore filtration, and added after the medium was autoclaved and cooled. The vitamins, when supplemented in concentrations greater than 1 mg/ml, were autoclaved together with the medium.

c. Preparation of synthetic media. The synthetic medium of Idriss and Blumenthal shown in Table 1, was sterilized by filtration

Table 1

COMPOSITION OF SYNTHETIC MEDIUM FOR THE GROWTH OF STAPHYLOCOCCI

Component	mg/L	Component	mg/L
Eagle's essential amino acids+			
L-Arginine	105.0.	Xanthine	0.5
L-Cystine	24.0	Ca pantothenate	0.1
L-Histidine	31.0	Pyridoxine-HCl	0.1
L-Isoleucine	52.4	$(NH_4)_2Fe(SO_4)2.H_2O$	14.5
L-Leucine	52.5	Riboflavin	0.1
L-Lysine	58.0	Biotin	0.005
L-Methionine	15.0	Folic acid	0.005
L-Phenylalanine	32.0	Vitamin B12	0.01
L-Threonine	49.0	Niacin (nicotinic acid)	8.0
L-Tryptophane	10.0	L-Glutamine	2,000.0
L-Valine	46.0	D-Glucose or sodium pyruvate	20,000.0
L-Tyrosine	36.0		
Eagle's non-essential amino acid*		Hank's salt solution to 1,000 ml	
L-Alanine	1,780.0	NaCl	8,000.0
L-Asparagine.H_2O	3,000.0	KCl	400.0
L-Aspartic acid	2,660.0	$Na_2HPO_4.2H_2O$	60.0
L-Glutamic acid	2,940.0	KH_2PO_4	60.0
L-Proline	2,300.0	$MgSO_4.7H_2O$	100.0
L-Serine	2,100.0	CaCl (anhyd.)	140.0
Glycine	1,500.0	$MgCl_2.6H_2O$	350.0
pH adjusted to 7.0 with about 40 ml of 0.5 M K_2HPO_4			

+ Eagle's essentail amino acid (minus glutamine) added as 20 ml of a 50-fold concentrate

* Eagle's nonessential amino acids added as 20 ml of a 100-fold concentrate.

through a HA (pore size 0.45 micron) Millipore filter (Millipore Filter Corp., Bedford, Mass.), instead of autoclaving in order to avoid the unnecessary degradation of various components of the synthetic medium. Essential and non-essential amino acid concentrates were obtained from Grand Island Biological Co., Grand Island, N. Y. The whole filter unit and the flasks used to collect the filtrates were sterilized by autoclaving at 121 C for 15 min.

d. Preparation of vitamins. Niacin, niacin analogs, and thiamine solutions were prepared separately and sterilized by membrane filtration. A sterile Millipore "Swinnex" filter containing a membrane with a pore size of 0.45 micron was placed on the tip of a 30 ml Luer-lok syringe. The vitamins were added separately to the autoclaved medium after it had cooled to room temperature.

e. Growth conditions. A 100 ml of Vitamin-free Casitone or synthetic medium was inoculated with approximately 1 ml of starter culture medium (Vitamin-free Casitone) that had been inoculated, and incubated on a shaker for 6-7 hr as described previously. The inoculated flasks were incubated at 37 C on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N. J.) at 240 rev/min. After 17 hr of growth, when the cells just started to enter into stationary phase, the cells were harvested by centrifugation at 0-5 C at

10,000 X g for 15 min.

f. Incubation of washed-cell suspension. In order to harvest cells for the incubation experiments, all steps including the use of sterile containers and solutions were performed aseptically. The 0.05 M potassium phosphate buffer, pH 7.0 used for incubating washed-cell suspensions was autoclaved at 121 C for 20 min. One gram of powdered glucose was added immediately to 100 ml of the potassium phosphate buffer after it had been removed from the autoclave. After cooling the glucose containing buffer, the washed-cell suspension of staphylococci was added to the buffer which was kept at room temperature, and the cell density in the buffer was adjusted to approximately 0.6 mg dry wt./ml, by using a Klett-Summerson colorimeter. For niacin uptake studies, staphylococci were incubated in 0.05 M potassium phosphate buffer, pH 7.0, 0.05 M glucose, and 50 µg/ml of niacin. To deplete the cells of nicotinamide coenzymes, washed-cell suspension of staphylococci was incubated in the absence of niacin.

g. Dry weight of staphylococcal cells. The dry weight of cells was determined by placing the washed cells in distilled water onto aluminium pans and weighing them after they had dried in an oven for 24 hr at 120 C.

h. Wet weight of staphylococcal cells. The wet weight of cells was determined by substracting the tare weight of a 30 ml

Corex centrifuge tube after centrifuging of the cells in the tube, with the elimination of excess water.

1. Turbidimetric measurement. Turbidimetric measurements were made with a Klett-Summerson colorimeter with a red filter (no. 60). The pellet was suspended in 0.8% saline. A reading of 195 Klett units was equivalent to 0.33 mg/ml dry wt. Multiplication of this value with the dilution factor gave the original dry weight of the cell suspension. When measuring a growth curve, the turbidity measurements were usually taken at hourly intervals using the side-arm nephelometric flasks.

B. Preparation of labelled pyridine-N-oxide. The preparation of tritium labelled pyridine-N-oxide was essentially the method of Ochiai (93) used for unlabelled compounds. To a solution of 1 ml of pyridine generally labelled with ^{3}H (1.2 mCi; 151 mCi/mM) in 1 ml of glacial acetic acid, 1 ml of 35% hydrogen peroxide was added dropwise into a round-bottomed flask and the mixture was refluxed in a water bath at 70-80 C for 24 hr. The mixture was neutralized with 1 N NaOH, diluted with 10 ml of water, and the solution was concentrated in a flash evaporator at 50 C. Both hydrogen peroxide and excess acetic acid were easily removed within the first 20 min. The evaporation was stopped when no more hydrogen peroxide could be detected. For the test,

a small sample was removed from the flask and the absence of gas evolution upon the addition of a small amount of solid palladium carbon indicated the absence of hydrogen peroxide.

C. Extraction and determination of nicotinamide nucleotides and adenine nucleotides.

a. Sampling of staphylococcal cells. Staphylococcal cells that had been grown in Vitamin-free Casitone with or without vitamin supplement were harvested when the cells had just entered the stationary phase. The cells were sedimented in 250 ml centrifuge bottles at 10,000 x g for 10 min in a B-20 refrigerated centrifuge. The supernatant fluid was quickly decanted, and the packed cells were resuspended in approximately 10 ml of chilled 0.8% saline and transferred to a tared 30 ml Corex centrifuge tube. The cells were recentrifuged, the supernatant fluid decanted, and the tube containing the packed, washed cells was stored in ice until ready for extraction.

b. Extraction of staphylococcal nucleotides. The method of extraction of the adenine nucleotides was essentially that of Neuhoff (88). The cells were suspended in a buffered extraction reagent containing 0.04 M Tris-HCl, pH 7.3, 3.5×10^{-3} M sodium EDTA, and 5×10^{-3} M cysteine hydrochloride, with the final pH of the solution adjusted to pH 7.5. Since the nucleotides are relatively unstable (14), all

steps in the extraction procedure were done in ice bucket at 0-5 C and at close to neutral pH, in a 30 ml Corex centrifuge tube. About 1 gram (wet weight) of cells was suspended in 10 ml of Tris-EDTA-cysteine reagent. The undiluted liquid phenol (89%) was added dropwise to the cell suspension over a period of 1-2 min until the extract was saturated with phenol, while it was being stirred vigorously with a vortex mixer. Since it was difficult to determine the point of saturation with the cells present, the necessary amount of phenol needed was predetermined with buffer. Approximately 1 ml of phenol was required to saturate to 10 ml of the buffer. The phenol-containing cell suspension was then treated with 20 ml of chloroform to remove the phenol. The cell suspension was centrifuged for 10-20 min at 20,000 x g and the upper aqueous phase was transferred to another chilled 30 ml Corex centrifuge tube. This was then treated with chloroform a second time, and after recentrifugation, the aqueous phase was removed with a pipette and added to a test tube kept in ice. The spectrophotometric enzymatic assays for the nicotinamide and adenine nucleotides were immediately performed using the above extract.

c. Extraction of staphylococcal nucleotides for anion exchange column chromatography. Perchloric acid extraction of

nucleotides from staphylococci was routinely employed when the cell extract was to be used for column chromatography. However, phenol extraction of cells suspended in distilled water was also used on occasions, yielding the same results. Approximately 5 volumes of 0.6 N perchloric acid was added to 1 g (wet weight) of washed cells and extracted by mixing intermittently with a Vortex Jr. mixer for 5-10 min. The cell suspension was then centrifuged for 10 min at 20,000 x g and the supernatant fluid was neutralized. Neutralization of the extract was performed by slow addition of 3 N KOH, while the extract was vigorously stirred with a magnetic stirrer, until the pH was 7.5 as measured with a pH meter. The insoluble potassium perchlorate precipitate in the extract was removed by centrifugation and the extract was immediately used for column chromatography.

d. Anion exchange column chromatographic procedures. The isolation and separation of labelled or unlabelled coenzymes from the perchlorate acid extract was accomplished by anion exchange chromatography in the following fashion. First, the Dowex-1 X 8 (Cl^-), 200-400 mesh resin was washed with deionized distilled water, and after most of the resin had settled, the fine particles were decanted and discarded. The resin was then washed twice with an equal volume of 1 N NaOH, followed by two washings with an equal volume of

1 N HCl. After washing the resin several times with distilled water, it was then suspended in 3.5 M sodium formate, and left overnight. To pack the column, the Dowex resin was resuspended in distilled water, and poured into a 25 by 1.1 cm chromatographic column (Ace Glass Inc., N. J.). The resin was allowed to pack by gravity up to 20 cm in height, and was further converted to the formate form by washing with 100 ml of 3.5 M ammonium formate solution. The excess formate was removed from the column by washing with 200 ml of distilled water. The neutralized extract was lyophilized and then up to one third its original volume with water. The sample was added slowly to the Dowex-1 (formate) column and the sample washed into the column with 20 ml of water. The column contents were then eluted with a discontinous formic acid gradient. The 250 ml mixing flask, which was directly connected to the column and stirred with a magnetic stirrer, was filled with 200 ml of deionized distilled water, and the reservior connected to it was filled with 200 ml of 2 M formic acid. When the first batch of formic acid eluant was exhausted, a second batch of formic acid of higher molarity was used. The reservoir was next filled with 200 ml of 8 M formic acid and 1 M ammonium formate, and the mixing flask was filled with 200 ml of 0.5 M formic acid. The column eluates were continually monitored by the passage through a 97290 liquid micro- aperture

flowcell, and read at 260 nm with a Gilford Instrument Co., Model 2000 recording spectrophotometer. The flow rate was adjusted to 1 ml/min, and 10 ml fractions were collected using a Buchler automatic fraction collector. Appropriate fractions were pooled, and evaporated to dryness, and at 40 C with a flash evaporator. The residue in the evaporating flask was taken up in 1 ml of 0.005 M potassium phosphate buffer, pH 7.5, lyophilized, and then resuspended in 0.1 ml of distilled water. After spotting the samples onto Whatman # 1 chromatography paper, the chromatograms were developed overnight by the descending technic with one of the following solvents: solvent 1. n-propanol saturated with water; solvent 2. absolute ethanol - 1 M ammonium acetate, pH 7.5 (7:3); solvent 3. isobutyric acid - conc. ammonium hydroxide - water (66:1:33), pH 3.7; solvent 4. 0.2 M potassium phosphate buffer, pH 6.8, and 6 M ammonium sulfate - n-propanol (100:60:2).

e. Chromatography. Whatman # 1 chromatographic paper was used throughout. Either ascending or descending chromatography was run at room temperature. The solvents were allowed to evaporate at room temperature before the papers were observed under UV light.

f. Estimation of adenine nucleotides. All spectrophotometric assays were performed at 30 C with a Gilford Model 2000

spectrophotometer (Gilford Instrument Co., Oberlin, Ohio), with an automatic sample changer and recorder. Standard 1.5 ml quartz cuvettes with a 1 cm light path were used as blanks and standard curves with known amounts of each cofactor were constructed. The procedures for the determination of the different nucleotides, as outlined in Table 2, is essentially that described in the Bergmeyer book.

g. Detection and characterization of nicotinamide nucleotides. An ultraviolet (UV) lamp, Model UVSI 13 (Ultraviolet Products Inc., San Gabriel, Calif.) was used for the detection of nucleotides after paper chromatography. Nicotinamide nucleotides were further identified by the development of UV fluorescent spots after exposing the chromatogram to the vapor of methylethylketone, and concentrated ammonium hydroxide (1:1, V/V). Nicotinic acid nucleotide could be distinguished from the nicotinamide nucleotide by fluorescence of the latter after exposure to methylethylketone and ammonia. Nicotinic acid and nicotinamide were identified on paper by the development of orange-yellow spots after exposure to cyanogen bromide vapor in a closed tank for 4 hr, followed by spraying with 2% p-aminobenzoic acid in 25% alcoholic 0.1 N HCl (66). Radioactive nucleotides which had been separated by paper chromatography were counted in a Packard Tri-carb liquid scintillation spectrometer, Model

Table 2

ESTIMATION OF ADENINE NUCLEOTIDES IN STAPHYLOCOCCAL CELL EXTRACT

Ref.	Subs.	Cell taken (mg dry wt)	First additions (reaction mixt)	Second additions	Third additions
72	ATP	1.0	Tris-buffer, pH 7.5 (100 mM); NADP (0.03 mM); $MgSO_4$ (5 mM); extract; read A_0.	Yeast G6PD (5 ug/ml) read A_1 after 10 min+ $A_3 - A_2$ = ATP	Glucose (1 mM); read A_2; then add yeast HK (5 µg/ml); read A_3 after 20 min.
1	ADP	1.0	Tris-buffer,pH 7.5 (100 mM); $MgSO_4$ (5 mM); PEP (0.04 mM); NADH (0.015 mM); extract; read A_1.	Muscle LD (PK free) 10 µg/ml;read A_1* after 10 min.	Muscle PK (5 µg/ml) : read A_2 after 15 min. $A_2 - A_1$ = ADP
1	AMP	1.0	''	'' $A_3 - A_2$ = AMP	Muscle PK;read A_2, then add muscle MK (5 µg/ml);read A_3 after 20 min.
62	NAD	1.5	Tris-HCl,pH 7.5 (100 mM) ethanol (0.01 ml); extract; read A_0.	Yeast ADH (10 µg/ml).	read A_1 after 15 min.
62a	NADP	1.5	Tris-HCl,pH 8.5 (100 mM); $MgSO_4$ (5 mM); G6P (1 mM); extract; read A_0.	Yeast G6PD (5 µg/ml)	read A_1 after 15 min.

+, determination of G6P; *, determination of pyruvate;

A_0, first absorbancy reading; A_1, second absorbancy reading; A_2, third absorbancy reading; A_3, fourth absorbancy reading.

3320. Samples were prepared for counting by cutting into small pieces a 4 X 6 cm section containing the radioactive material from the chromatogram. The paper sections were then placed in a scintillation vial and 15 ml of the scintillation fluid was added to the vial. The scintillation fluors contained the following reagents: 1,4-dioxane, 900 ml; anisole, 150 ml; 1,2-dimethyoxyethane, 150 ml; 2,5-diphenyloxazole (PPO), 18 g; and 1,4-bis-(2-(5-phenyloxazolyl))-benzene (POPOP), 60 mg (21). Control sections of an equal size were also cut from the chromatogram and counted in the same manner.

D. Materials.

a. Chemicals and enzymes. The chemicals and enzymes used were obtained from commercial sources, and were not further purified. β-NAD, NADP, β-NADH, NADPH, AMP, ADP, ATP, G6P (sodium), sodium pyruvate, puromycin (dihydrochloride), phosphoenolpyruvate (tricyclohexylammonium salt), folic acid, crystalline L-chloramphenicol, muscle lactic dehydrogenase (Type II), yeast G6PD (Type II), yeast hexokinase (Type III), rabbit muscle pyruvate kinase (Type II), rabbit myokinase, and bovine liver glutamic dehydrogenase (Type I), were purchased from Sigma Chemical Co., St. Louis, Mo.. Niacin (nicotinic acid) was obtained from Merck and Co., Rahway, N. J.; biotin, xanthine, and α-ketoglutarate were

from Nutritional Biochemical Corp., Cleveland, Ohio; actinomycin D was obtained from Mann Res. Lab., N. Y., N. Y.. PPO (2,5-diphenyloxazole, scintillation grade), anisole, and 1,2-dimethyloxyethane were obtained from Fisher Scientific Co., Fair Lawn, N. J.. POPOP (scintillation grade) was obtained from Packard Instrument Co., Downers Grove, Ill.. All other chemicals were reagent grade with the exception of U. S. P. liquified "Gilt Label" phenol (Mallincrodt, St. Louis, Mo.).

b. Niacin analogs. Ethylnicotinate, 3-hydroxypyridine-N-oxide, 2-chloronicotinic acid, isonicotinic acid hydrazide, quinolinic acid, nicotinic acid-N-oxide, nicotinamide-N-oxide, thionicotinamide, N-methylnicotinamide, 6-chloronicotinamide, 6-hydroxynicotinic acid, 3-picoline-N-oxide, 2-picoline-N-oxide, pyridine-N-oxide, 3-acetylpyridine, 3-pyridylcarbinol, 3-pyridylcarbinol-N-oxide, ethylnicotinate, pyridylsulfonic acid, 5-chloronicotinic acid, N-ethylnicotinamide, 2-aminonicotinic acid, pyridylacetic acid, picolinic acid, 6-chloronicotinamide, dipicolinic acid, 3-benzoylpyridine, 3,4-diaminopyridine, 2,4-pyridine dicarboxylic acid, pyridine, 4-acetylpyridine, pyridoxine, 2-picoline, 4-hydroxynicotinic acid, pyrizinamide, 3-hydroxypicolinic acid, N,N-diethylnicotinamide, pyridine-3-aldehyde, pyridine-2-aldehyde, 6-aminonicotinamide were obtained from Aldrich Chemical

Co., Milwaukee, Wis.; 6-hydroxynicotinic acid, isonicotinic acid, β-picolinic acid were obtained from Mann. Res. Lab., N. Y., N. Y..

c. Radiochemicals. Nicotinic acid (carboxyl-^{14}C) 27.9 mCi/mM was obtained from Nuclear Chicago, Chicago, Ill., isoniazid-^{14}C (carbonyl-^{14}C) 9.85 mCi/mM, and methionine (methyl-^{14}C) 60 mCi/mM, quinolinic acid-6-^{14}C (29.3 mCi/mM), amd pyridine-^{3}H(G) 151 mCi/mM were obtained from Amersham/Searle, Chicago, Ill. Glycerol-UL-^{14}C (18 mCi/mM), formaldehyde-^{14}C (1.5 mCi/mM) and succinic acid-2,3-^{14}C (ca. 5.9 mCi/mM) were obtained from the International Chemical and Radioisotopes Corp., Irvine, Cal..

RESULTS

A. Effect of niacin on the growth, coenzyme content, and glycolytic pathways.

a. Growth and nucleotide levels of S. aureus grown in thiamine supplemented Vitamin-free Casitone medium containing different concentrations of niacin.

The effect of exogenous niacin on the growth, the total amount of NAD(P), and the free adenine nucleotides were closely examined in staphylococci. An examination of the data in Table 3 indicates that the growth, and the total amount of niacinamide nucleotides synthesized were affected by the amount of niacin added to the growth medium. On the whole, there was a larger increase in NAD content from 0.3 to 5.7 μmole/ g dry wt.) than NADP (from 0.2 to 0.6 μmole/g. dry wt.), when increasing amounts of niacin were included in the growth medium. In the range of 0 to 2 μg/ml of niacin added to the growth medium, the total amount of NAD(P) in staphylococci was somewhat proportional to the amount of exogenous niacin added. Staphylococci grew poorly, and reached a turbidity of only 100 Klett units when grown in Vitamin-free Casitone, even with a thiamine supplement. The cells had low content of NAD (0.3 μmole/g), and of NADP (0.2 μmole/g). The addition of 0.1 μg niacin/ml to the growth medium resulted in maximal staphylococcal cell yield of 365 Klett units

Table 3

GROWTH AND NUCLEOTIDE LEVELS IN S. AUREUS GROWN IN THIAMINE SUPPLEMENTED VITAMIN-FREE CASITONE CONTAINING DIFFERENT CONCENTRATIONS OF NIACIN

Niacin added (µg/ml)	Turbidity Klett units	NAD	NADP	AMP	ADP	ATP
			µmole/g. dry wt.+			
0	100	0.3	0.2	2.1	1.1	.2.0
0.1	365	0.4	0.2	2.7	2.7	3.2
0.25	360	1.3	0.5	4.8	4.5	5.0
0.50	360	1.8	0.5	3.7	5.4	10.2
2	365	5.7	0.6	2.2	4.5	14.5
5	360	5.5	0.6	1.6	4.1	15.6
50	360	5.0	0.6	1.3	3.3	12.5
500	320	4.0	0.4	1.9	3.5	7.2
1000	160	3.0	0.4	2.1	2.5	6.1

+ Cells were grown for 17 hr in 2% Vitamin-free Casitone supplemented with 2 µg/ml of thiamine, and the indicated amount of niacin. The unsupplemented medium contains less than 0.013 µg/ml of niacin. Following growth, the nucleotides were extracted and assayed as described in Methods.

but there was no measurable increase in the cellular NAD(P) content. When 0.25 µg/ml of niacin was added to the Vitamin-free Casitone medium, there was maximal yield of cells, and the NAD content was 1.3 µmole, and the NADP content was 0.5 µmole/g. dry wt. This represented a 3.4-fold increase in NAD, and a 2-fold increase in NADP, as compared to cells grown in thiamine enriched Vitamin-free Casitone medium. However, staphylococci grown in 0.5 µg/ml of niacin in the medium had a NAD content which was only 24% of that found in cells grown in Vitamin-free Casitone medium supplemented with optimal amounts of niacin (2 µg/ml). A 3-fold increase in the operation of the HMP pathway was observed when the growth medium was supplemented with 0.25 µg/ml of niacin, but no increase when only 0.1 µg/ml of niacin was added. Thus, there was a concomitant increase in the total NAD(P) content, and the stimulation in the operation of the HMP pathway. The total NAD(P) content in staphylococci was 2.3 µmole/g. dry wt., when the medium was supplemented with 0.5 µg/ml of niacin. Staphylococci contained maximal levels of NAD(P), 6.3 µmole/g. dry wt., when 2 µg/ml of niacin was added, and these levels remained almost the same even when 5 µg/ml of niacin was added to the medium. These maximal NAD(P) levels, however, could be realized even at levels lower than 2 µg niacin/ml in the

medium.

Staphylococci grown in Vitamin-free casitone medium supplemented with thiamine had AMP, ADP, and ATP levels of 2.1, 1.1, and 2.0 μmole/g. dry wt. respectively, and with the ATP/AMP ratios close to 1 (Table 3). When the media were supplemented with optimal amounts of niacin (2 μg/ml), the ATP/AMP ratios were greater than 6. However, no direct correlation between the increase in NAD(P) and the adenine nucleotides could be observed in these cells. Staphylococci grown in Vitamin-free Casitone medium supplemented with optimal amounts of niacin, and thiamine always had a high ATP content.

b. Changes in adenine nucleotide levels during incubation of washed-cell suspensions of staphylococci in buffered glucose. The intracellular levels of adenine nucleotides during the course of incubation of washed-cell suspensions in buffered glucose are shown in Table 4. During the period of incubation, the NAD content dropped drastically, and after 3 hr, it had been reduced by 3.2 μmole/g. dry wt., representing over a 50% decrease. On the other hand, the level of NADP increased from 0.7 to 1.0 μmole, but representing a 43% increase over the initial value. The ATP, and ADP contents increased considerably during incubation in the presence of glucose. As would be expected, the ADP and ATP levels increased but the AMP level decreased. However, since the

Table 4

ALTERATION OF INTRACELLULAR ADENINE NUCLEOTIDES OF S. AUREUS DURING INCUBATION IN BUFFERED GLUCOSE

Cofactors	Nucleotide levels µmole/g. dry wt.+		
Hour	0	3	6
NAD	6.7	3.2	2.5
NADP	0.7	1.0	1.0
AMP	3.0	1.2	1.5
ADP	3.7	4.9	6.0
ATP	13.5	24.1	20.8

+ After growth for 17 hr in 2% Vitamin-free Casitone supplemented with 4 µg/ml of niacin, the cells were washed with, and suspended at twice their original concentration in 0.05 M potassium phosphate buffer, pH 7.0, containing 1% glucose. Cells were incubated at 37 C on a rotary shaker and samples were removed and analyzed after 0, 3, and 6 hr.

total amount of ADP, and ATP increased during the course of the 6 hr incubation period, the synthesis of some adenine nucleotides must have occurred from endogenous precursors. Cells grown in niacin supplemented Vitamin-free Casitone medium had an initial ATP/AMP ratio close to 5, while incubation in the presence of glucose, the ATP/AMP ratio exceeded 20. Also there was approximately a 10-fold increase in pyruvate. Since the cells were grown in thiamine deficient medium, the accumulation of pyruvate was expected. We found that the absolute content of NAD was approximately 10-fold higher than that of NADP, and the NAD level showed a greater degree of variation or decrease than NADP.

c. Biochemical changes induced by the addition of vitamins to vitamin-deficient S. aureus under conditions precluding protein synthesis. To eliminate the possibility that the increased amounts of NAD, NADP, and the HMP pathway and TCA cycle activities that were found following the addition of vitamins to vitamin-deficient resting cell suspensions were not due to new enzyme synthesis during incubation, the cells were incubated for 3 hr in buffered glucose containing niacin and thiamine, with or without one of the following antibiotics which inhibit protein synthesis: chloramphenicol, puromycin, or actinomycin D. The antibiotics were added to preclude the possibility of any protein synthesis that might be possible

Table 5

BIOCHEMICAL CHANGES INDUCED BY THE ADDITION OF VITAMINS TO VITAMIN-DEFICIENT *S. AUREUS* UNDER CONDITIONS PRECLUDING PROTEIN SYNTHESIS

	Vitamin-deficient resting cells incubated in buffered glucose containing niacin + thiamine and:+					Vitamin sufficient cells
	No antibiotic		Chloramphenicol, 25 µg/ml	Puromycin, 6 µg/ml	Actinomycin, 3 µg/ml	
	0 Hr	3 Hr	3 Hr	3 Hr	3 Hr	
Intact cells						
% glucose via HMP	5.0	16.0	14.0	18.5	34.0	16.0
Rel. act. of TCA	0.6	4.0	4.0	5.7	5.0	4.0
Cell extracts						
NAD, µmole/g dry wt.	0.2	3.5	3.8	3.5	13.2	6.7
NADP, µmole/g dry wt.	0.2	0.4	0.4	0.5	2.3	0.5
G6PD, Spec. act.*	46	42	42	-	-	145
ICD, Spec. act.*	22	13	16	-	-	58

+ Cells were grown for 17 hr in unsupplemented 2% Vitamin-free Casitone, harvested, washed with saline, and suspended in 0.05 M K phosphate buffer, pH 7, containing 0.9% glucose, niacin (8 µg/ml), and thiamine (4 µg/ml). Relative activities of the HMP and TCA cycle were determined by the yield of CO_2 from glucose-1-^{14}C and glucose-6-^{14}C respectively.

* Specific activity - nanomole of NADP reduced/mg of protein/min.

from the free amino acid pool in the washed cells. Thus, the new synthesis of some HMP or TCA cycle enzymes was eliminated with chloramphenicol, or actinomycin D. The results presented in Table 5 show that none of the antibiotics prevented the stimulation of NAD and NADP formation as a result of the addition of niacin and thiamine to the resting cells. The NAD content increased approximately 10-fold, while the NADP level increased 2-fold during the 3 hr incubation. Concomitant with the increase in coenzymes, the increased oxidation of glucose by the HMP pathway also was induced by niacin during incubation. However, the specific activities of the HMP, and other enzymes tested were unchanged, indicating that the changes in the enzyme levels were secondary to those of the nicotinamide coenzymes. Also, the absence of enzyme synthesis in the presence of chloramphenicol eliminated the possibility that preformed peptide precursors of enzymes might have been activated. These experimental data also indicated that staphylococci synthesized approximately 1 nanomole of NAD/mg cells/hr. Incubation of vitamin-deficient resting cells in the presence of thiamine alone has no effect on the NAD(P) content. Surprisingly, the presence of actinomycin D actually stimulated coenzyme synthesis.

d. <u>Effect of varying intracellular concentrations of NAD(P) on the glycolytic pathways</u>. The previous experiments indicated

that the relative amounts of cellular coenzymes in staphylococci could be adjusted somewhat by the level of niacin added to the growth medium. We then attempted to devise an experiment in which the level of intracellular NAD(P) was just high enough to allow for maximal operation of the HMP pathway. This was achieved by providing 0.5 µg/ml of niacin to the growth medium. The NAD level of such cells could then be reduced by incubation of the cells in buffered glucose (see Table 4). The results of such an experiment, and of the HMP pathway, and the TCA cycle activities of the resulting cells, are presented in Fig. 1. The freshly harvested cells contained 2.2 µmoles of NAD and 0.45 µmole of NADP/g. dry wt., and the cells exhibited maximal operation of both the HMP pathway (17.3%), and the TCA cycle. Following the first 2 hr period of incubation in buffered glucose, there was a decrease of NAD from 2.2 µmole to 1.0 µmole, and there was also a decrease of the operation in the HMP pathway; the TCA cycle activity remained relatively unchanged. However, there was no change in the NADP level during this 2 hr period, during which glucose oxidation through the HMP pathway had decreased by 50%. Clearly then, the level of NADP was not responsible for the decreased HMP activity of the pathway. Prolonged incubation led to further decrease in the cellular NAD level until it reached 0.5 µmole/g. dry wt. at the end of 6 hr. By this time,

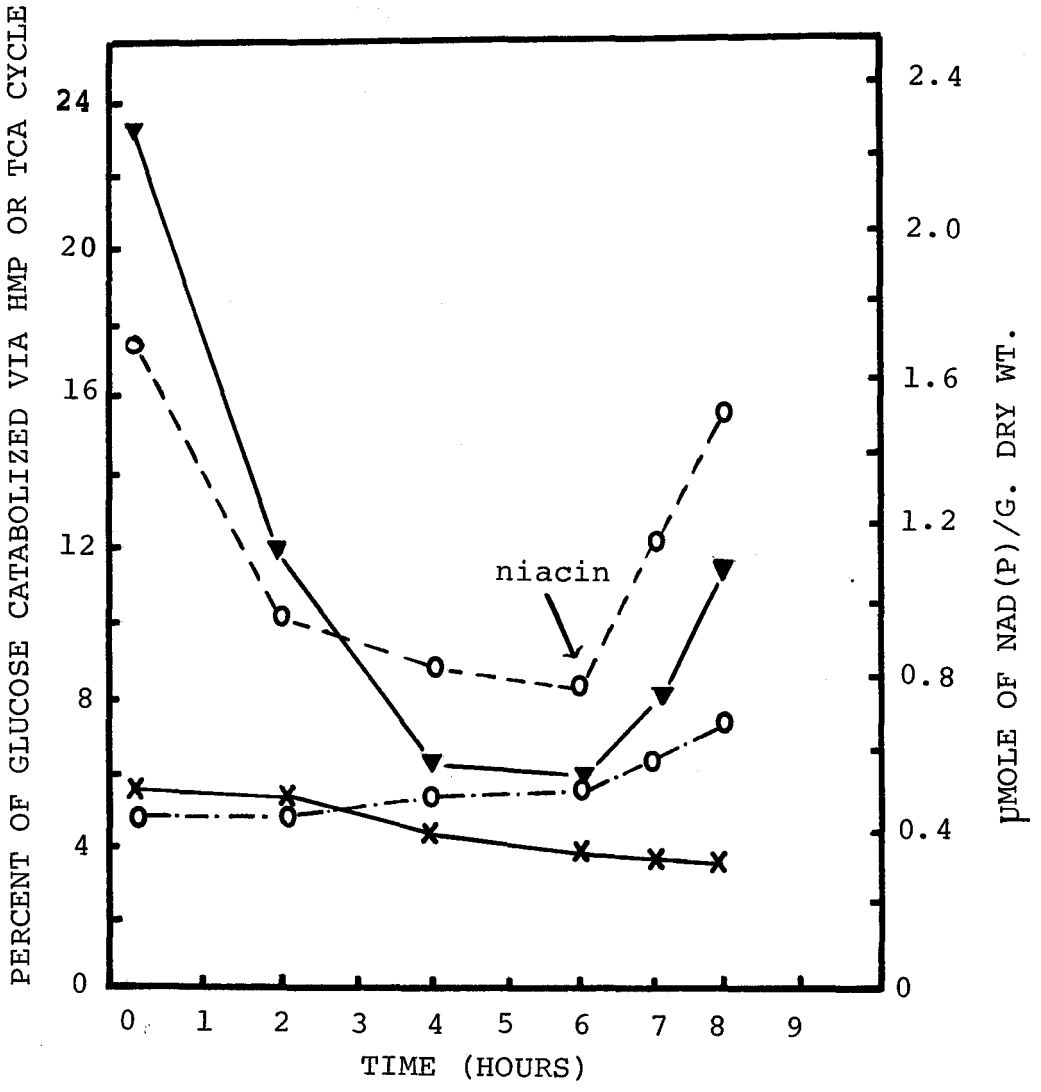


Fig. 1. Biochemical changes occurring in S. aureus during incubation in buffered glucose.

Cells were grown for 17 hr in 2% VFC medium supplemented with 0.5 μg/ml niacin and 4 μg/ml thiamine, harvested, washed with saline, and incubated in 0.05 M K phosphate buffer, pH 7, containing 1% glucose. After 6 hr incubation, 100 μg/ml of niacin was added to the cell suspension and further incubated for 2 hrs. At intervals, samples were removed, and extracted for coenzymes, and the relative HMP and TCA cycle activities were determined by the yield of CO_2 from glucose-1-^{14}C and glucose-6-^{14}C respectively (36). o–o, HMP; ×–×, TCA; ▼–▼, NAD; o···o, NADP.

the NADP level had actually increased slightly. At this point, the administration of 100 μg/ml of niacin resulted in a relatively rapid increase in the NAD level, and a concomitant increase in the HMP pathway activity. One hr after the addition of niacin, the NAD content increased from 0.5 to 0.7 μmole, while the HMP pathway also increased its activity from 8% to 12%; the TCA cycle remained unchanged.

B. Effect of niacin analogs on the growth, coenzyme content, and glycolytic pathways.

a. Effect of niacin analogs on growth stimulation. The growth curves of S. aureus Towler in Vitamin-free Casitone medium supplemented with various niacin analogs, with or without thiamine, are shown in Fig. 2 and Fig. 3. The Vitamin-free Casitone (C) medium without the addition of any vitamin, supported a moderate level of staphylococcal growth. This was due to the fact that a 2% solution of the so-called Vitamin-free Casitone contained a low and somewhat variable amount of niacin (<0.0132 μg/ml), and thiamine (<0.00013 μg/ml). These values were provided by the Difco Laboratories. The addition of 8 μg/ml of niacin to the growth medium led to more than a 2-fold increase in cell yield. However, any of the three niacin analogs that were added to the medium, 3-acetylpyridine (1000 μg/ml), nicotinyl diethylamide (1000 μg/ml), or nicotinamide-N-oxide (100 μg/ml), resulted only in slight stimulation of growth..

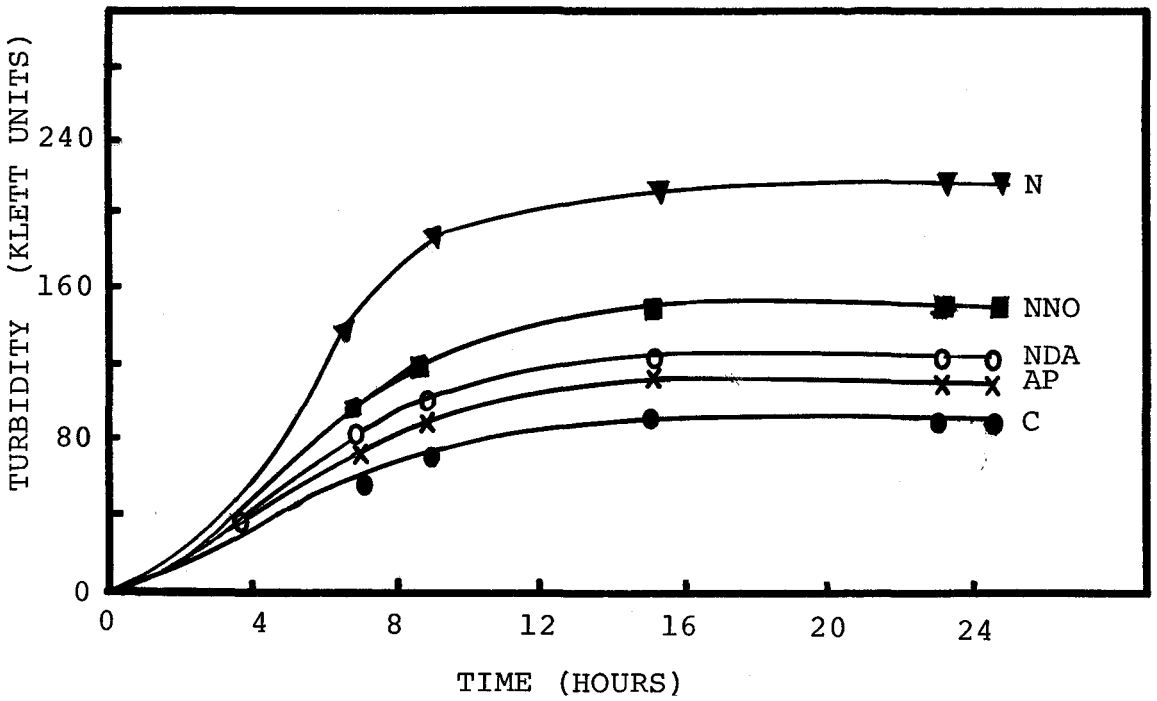


Figure 2. Effect of niacin or niacin analogs on the growth of S. aureus Towler in 2% Vitamin-free Casitone medium.

C-Control (no vitamin addition); N-niacin (8 µg/ml); AP- 3-Acetylpyridine (100 µg/ml); NDA- nicotinyl-diethylamide (100 µg/ml); NNO- nicotinamide-N-oxide (100 µg/ml); T- Thiamine (4 µg/ml).

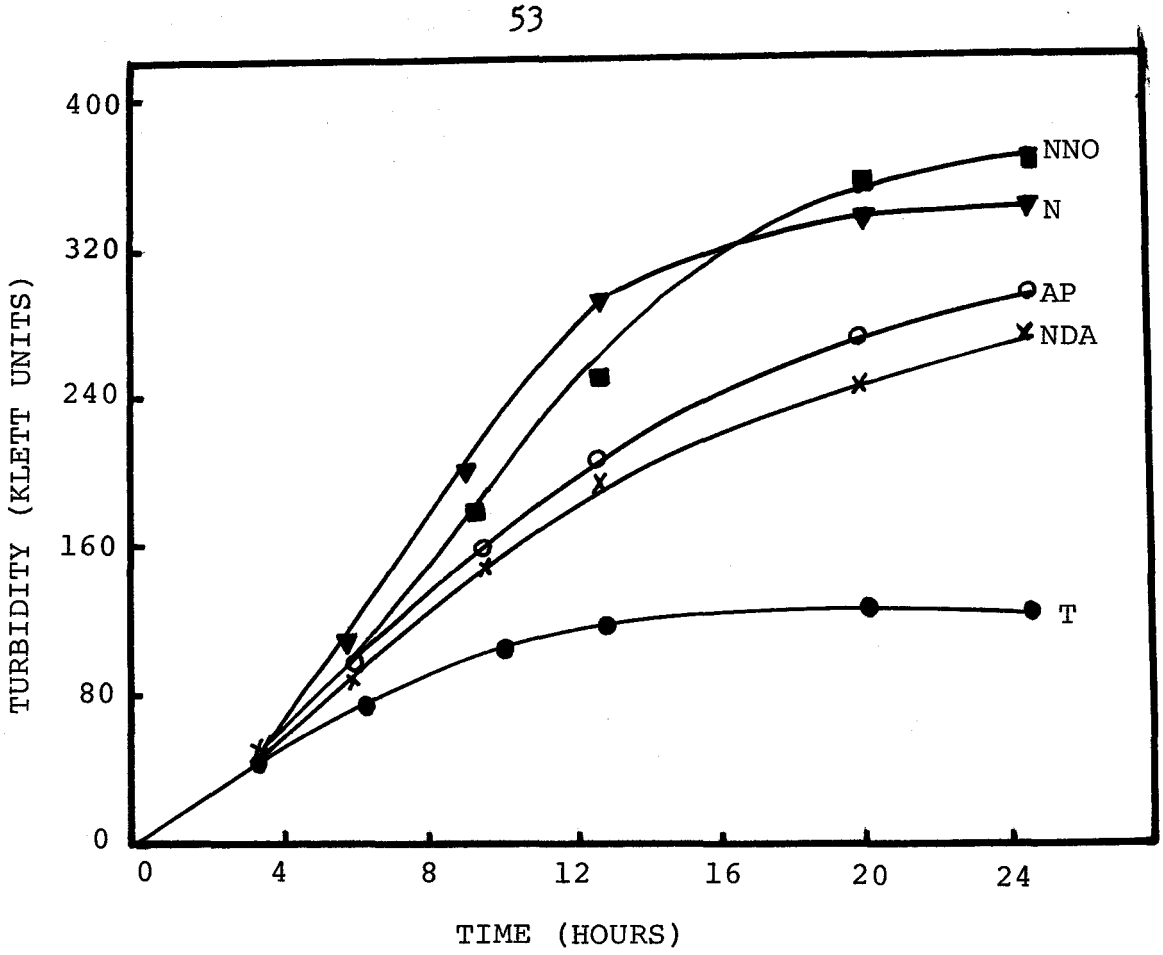


Figure 3. Effect of niacin or niacin analogs on the growth of S. aureus Towler in thiamine supplemented 2% Vitamin-free Casitone medium.

T-thiamine (4 µg/ml); N- niacin (8 µg/ml); AP- 3-acetyl-pyridine (1000 µg/ml); NDA- nicotinyl diethylamide (100 µg/ml); NNO- nicotinamide-N-oxide (100 µg/ml).

The addition of thiamine alone to the medium had little stimulatory effect on the growth of staphylococci, whereas the addition of both niacin and thiamine resulted in maximal cell yield; and there was a 3-fold increase in turbidity (Fig. 3). When the niacin analogs were added together with thiamine, there was also maximal cell growth. However, the growth rate with the analogs was somewhat slower, and the final cell yield usually less, than those cultures containing niacin; nicotinamide-N-oxide was an exception. Also the log phase of the growth curves with the analogs was more prolonged than that with cells grown in the presence of niacin. The growth curves indicated that growth with the niacin analogs does not involve the selection of mutants since no extended or variable lag period is observed in cells grown in the presence of these analogs.

b. Effect of niacin analogs on the growth of staphylococci and other niacin-requiring bacteria in a synthetic medium. To insure that the niacin analogs could replace niacin in more than one staphylococcal strain, we also tested 6 additional strains of S. aureus, 3 strains of S. epidermidis, and one strain each of the Gram-negative bacteria, Proteus vulgaris, Proteus morgani, and Shigella flexneri (Table 6). The growth of cells in the media supplemented with either 3-acetyl-pyridine (1000 µg/ml), or nicotinyl diethylamide (100 µg/ml),

Table 6

EFFECT OF NIACIN ANALOGS ON THE GROWTH OF STAPHYLOCOCCI AND OTHER NIACIN-REQUIRING BACTERIA IN A SYNTHETIC MEDIUM+

Strains	Niacin + thiamine		3-Acetylpyridine + thiamine		Nicotinyl-diethylamide + thiamine	
	Klett units after					
	24 Hr	48 Hr	24 Hr	48 Hr	24 Hr	48 Hr
S. aureus Towler	239	270	134	205	107	150
S. aureus 3	92	185	171	168	30	170
S. aureus 10	229	264	184	176	89	93
S. aureus 18Z	228	270	166	228	41	168
S. aureus serotype 1	276	274	189	184	131	173
S. aureus serotype 3	243	282	192	259	221	271
S. aureus serotype 4	155	264	215	224	174	225
S. epidermidis 155	117	228	110	234	44	160
S. epidermidis 12228	212	210	189	199	63	155
S. epidermidis 14990	220	215	192	272	172	180
Shigella flexneri	145	137	122	117	103	115
Proteus morgani	210	200	202	209	183	159
Proteus vulgaris	211	195	175	184	186	167

+ Staphylococci and Gram-negative bacteria were grown in the synthetic medium supplemented with 4 µg/ml of thiamine, and 8 µg/ml of niacin or 1000 µg/ml of 3-acetylpyridine, and or 100 µg/ml of nicotinyl diethylamide. Turbidity was measured after growth for 24 and 48 hr.

and thiamine was compared to the growth of cells grown in the presence of niacin (8 µg/ml), plus thiamine (8 µg/ml) in the synthetic medium. Growth was measured turbidimetrically after 24 and 48 hr. When the synthetic medium was supplemented with niacin and thiamine, maximal cell yield ranged from 137 to 282 Klett units with all the species tested. The results clearly indicate that many niacin-requiring bacteria are able to use niacin analogs as a growth factor. Both 3-acetylpyridine and nicotinyl diethylamide were able to replace the niacin requirement for the growth, although neither of these analogs gave cell yields equivalent to niacin supplemented medium. However, in other experiments reported later, we observed that higher cell yields could be attained when still higher concentrations of niacin analogs were added to the synthetic medium. In general, S. aureus strain grew to the greatest extent in this medium as compared to the S. epidermidis, Proteus, and Shigella species.

c. Effect of various niacin analogs on the growth of staphylococci. A larger number of niacin analogs were studied to determine whether they could replace niacin as a growth factor. The results of these studies are presented in Table 7. The amount of growth after 24 and 48 hr in the synthetic medium lacking niacin, with or without thiamine, was negligible. With the addition of niacin to the synthetic media, there

Table 7

EFFECT OF NIACIN ANALOGS ON THE GROWTH OF S. AUREUS TOWLER IN SYNTHETIC MEDIUM+

Niacin analogs (µg/ml)	Synthetic medium Klett units after 24 Hr	48 Hr	Synthetic medium + thiamine 24 Hr	48 Hr
None	2	6	5	15
Niacin (8)	55	54	239	270
Nicotinamide-N-oxide (100)	16	28	293	343
Nicotinuric acid (10)	8	39	226	271
Nicotinyl diethylamide (100)	13	24	107	150
Thionicotinamide (500)	15	28	254	315
3-Acetylpyridine (1000)	5	11	134	205
Pyridine-3-aldehyde (500)	13	21	190	300
Pyridine-3-sulfonic acid (250)	26	40	95	110
6-Hydroxynicotinic acid (500)	2	6	7	15
6-Aminonicotinamide (100)	3	3	4	10

+, S. aureus Towler was grown in the synthetic medium of Idriss and Blumenthal (Table 1), containing niacin or its various niacin analogs supplemented at concentrations as indicated above, and with or without thiamine. Turbidity was measured after 24 and 48 hr.

was approximately a 10-fold increase in staphylococcal growth whereas the presence of both niacin and thiamine resulted in maximal cell yield. In the absence of thiamine, a wide variety of these niacin analogs gave slight stimulation of growth but none of these compounds was as effective as niacin. However, in the presence of thiamine, all of the analogs mentioned in the table, except 6-hydroxynicotinic acid, and 6-aminonicotinamide, gave approximately a 3-fold increase in growth. In fact, 6-aminonicotinamide was found to be inhibitory to staphylococci. We observed that the levels of analogs required for maximal growth stimulation were generally much higher than the levels of niacin required. In these experiments, only nicotinuric acid was able to substitute for niacin at a very low concentration. With acetylpyridine, 1 mg/ml was needed as compared to 0.2 µg/ml for niacin for maximal growth in Vitamin-free Casitone, and probably less than 1 µg/ml in the synthetic medium.

d. Effect of varying concentrations of 3-pyridylcarbinol on growth, pyridine nucleotides, and glycolytic pathways. In the course of the previous experiments, it became apparent that the NAD levels varied somewhat as the concentrations of niacin analogs were changed. The results of a representative experiment which shows the effect of the variation of 3-pyridylcarbinol (PC) concentration in the medium on growth,

nucleotide levels, and the HMP pathway are shown in Fig. 4. Vitamin-free Casitone supplemented with thiamine, but lacking niacin or a niacin analog such as PC, provided a poor medium for staphylococcal growth. Cells grown in this medium had intracellular NAD and NADP concentrations of only 0.4 and 0.2 μmole/g. dry wt., respectively. Furthermore, in these cells, there were low levels of the operation of both the HMP pathway and TCA cycle. When PC was added to the growth medium at a concentration of 2 μg/ml, there was no significant change in the coenzyme levels, and the HMP pathway, but there was a slight stimulation of growth. However, when 10 μg/ml of PC was added, there was maximal growth stimulation (350 Klett units) although the total amount of NAD and NADP remained relatively the same as in the cells grown in unsupplemented Vitamin-free Casitone medium. In other words, growth was maximal but NAD(P) levels were minimal, and the percentage of glucose oxidized via the HMP pathway was minimal. However, when the concentration of PC added to the medium was increased to 50 μg/ml, the staphylococcal cell yield increased no further, but the HMP pathway actually was increased 2.5-fold. Concomitant with the increase in HMP pathway, the NAD level increased 3-fold and the NADP level increased 2-fold. These results demonstrated that the operation of the HMP pathway was

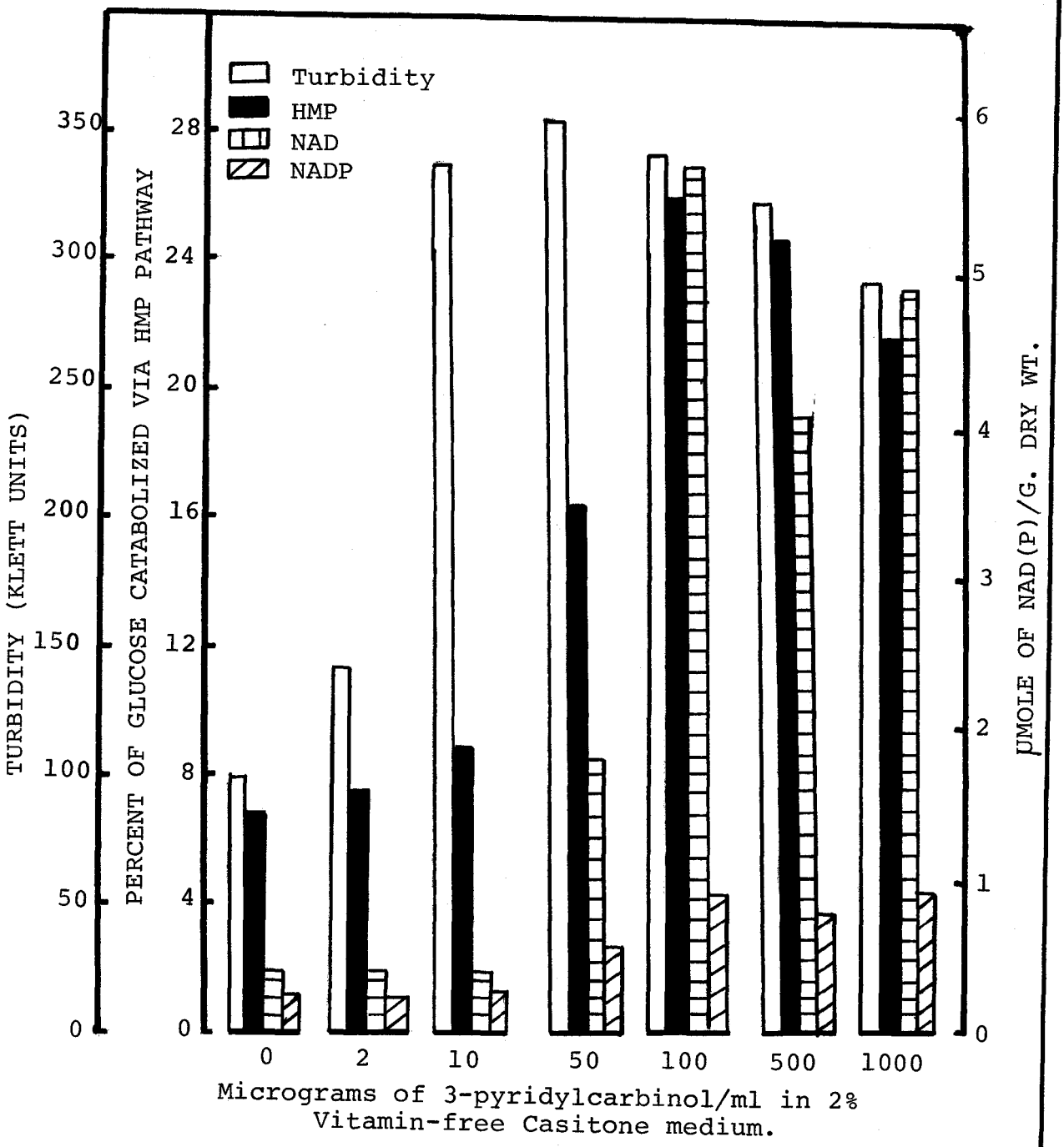


Figure. 4. Effect of varying concentrations of 3-pyridylcarbinol (PC) on growth, NAD(P) levels, and percent of glucose oxidation by the HMP pathway.

related to the level of the intracellular NAD and/or NADP. When 100 µg/ml of PC was added to the medium, there was no further increase in cell yield although the amount of glucose oxidized by the HMP pathway was stimulated 4-fold (to 27%). At the same time, the NAD level increase 12-fold (to 5.8 µmole) and the NADP concentration increased 6-fold (to 0.9 µmole). With still higher concentrations of PC, there was no further increase in the HMP pathway activity or the NAD(P) concentration. In other experiments (not shown), the same findings were reported with niacin and many other niacin analogs. A concentration of niacin, or niacin analog could be found that would yield maximal growth, although the levels of NAD and NADP were low, and the activity of the HMP pathway was low. At a 5- to 10-fold higher concentration of niacin or niacin analog, the NAD and NADP concentrations, and the HMP pathway activity was maximal. In the case of niacin, maximal growth was reached in the Vitamin-free Casitone medium when 0.05 µg/ml of niacin was added, whereas maximal NAD(P) levels, and the maximal levels of glucose oxidation by the HMP pathway required 1 µg/ml of niacin in the growth medium.

e. Effect of niacin analogs on the NAD(P) content of S. aureus. The NAD and NADP contents of S. aureus strain Towler grown in Vitamin-free Casitone media supplemented with thiamine and niacin, or various niacin analogs at their required

concentrations are shown in Table 8. Staphylococci grown in unsupplemented Vitamin-free Casitone, or medium supplemented with thiamine alone, had NAD and NADP levels of 0.3 to 0.4 umole, and 0.2 μmole/g. dry wt., respectively. The addition of niacin to the thiamine supplemented medium increased the NAD level 12-fold, and NADP level 3-fold. In other experiments not shown in the table, the NAD(P) levels were relatively the same whether the cells were grown in the presence or absence of thiamine. As shown in Table 4, the increased coenzyme content was also accompanied by a 3- to 4-fold increase in the amount of glucose oxidized by the HMP pathway. The increased NAD(P) contents of the cells grown in the presence of niacin analogs were harder to detect and were somewhat proportional to the amount of the analogs added to the media. In Table 8, the niacin analogs were listed in the decreasing order of their relative effectiveness in replacing niacin as a growth factor. It was found that the concentrations of analogs required for maximal growth were 20- to 30-fold higher than the concentration of niacin necessary to provide maximal cell growth. In general, when cells were grown in the presence of niacin analogs, the increased content of NAD(P) was paralleled by an increased ability of the cells to oxidize glucose via the HMP pathway. However, with certain analogs, such as 3-acetylpyridine or pyridylsulfonic acid, the stimulation of the HMP

Table 8

EFFECT OF NIACIN ANALOGS ON THE NAD(P) CONTENTS OF *S. AUREUS* +

Niacin analog added	Conc, µg/ml	Molar conc.	NAD µmole/g. dry wt.	NADP µmole/g. dry wt.
None (no thiamine)*	-	-	0.30	0.20
None (with thiamine)	-	-	0.40	0.20
Niacin	4	3.2×10^{-5}	4.80	0.60
Niacinamide	10	8.3×10^{-5}	5.70	0.63
Nicotinuric acid	8	4.9×10^{-5}	3.50	0.40
Nicotinamide-N-oxide	100	7.3×10^{-3}	5.10	0.75
Ethylnicotinate	10	6.6×10^{-3}	2.56	0.47
N-Methylnicotinamide	100	7.3×10^{-3}	6.18	0.69
3-Pyridylcarbinol	100	9.2×10^{-3}	5.70	0.94
Nicotinyl diethylamide	100	6.4×10^{-4}	4.14	1.15
Picoline-N-oxide	1000	9.2×10^{-3}	1.00	0.41
Isonicotinic hydrazide	1000	7.0×10^{-3}	1.94	0.45
Pyridine-N-oxide	3000	3.1×10^{-2}	1.70	0.43
Quinolinic acid	2000	1.2×10^{-2}	3.00	0.38
3-Acetylpyridine	5000	2.5×10^{-2}	1.50	0.18
3-Pyridylsulfonic acid	5000	3.6×10^{-2}	2.15	0.40
6-Hydroxynicotinic acid	500	3.6×10^{-3}	0.40	0.30
6-Aminonicotinamide	500	3.8×10^{-3}	0.20	0.20

+ *S. aureus* strain Towler was grown for 17 hr in 2% VFC medium supplemented with thiamine (4 µg/ml), and niacin analogs at the indicated concentrations. Vitamins and concentrations of analogs below 1 mg/ml were sterilized by membrane filtration and added to the media after they were autoclaved. Higher concentrations of analogs were added to the media before autoclaving.

* Unsupplemented medium with no thiamine added.

pathway activity by the analogs was more easily detected than the increased NAD(P) content. The coenzyme contents were higher with those niacin analogs that stimulated maximal cell growth at relatively low concentrations (less than 100 µg /ml or 10^{-4} M). Pyridine derivatives, with substituents in the 3 position, such as the pyridylcarbinol, pyridylaldehyde, or nicotinuric acid, are all in this category. Substitution of pyridine at positions other than 3-position usually resulted in a lesser amount of the coenzyme formed. 3-Pyridylcarbinol, added as a supplement at 100 µg/ml (9.2×10^{-3} M) to the growth medium resulted in a 15-fold increase in NAD whereas 3-acetylpyridine at the same concentration did not increase the coenzyme level. Nicotinuric acid was as effective as niacin in stimulating the HMP pathway and increased coenzyme levels when given in 10-fold higher concentration than niacin. Since this compound is merely a peptide of glycine and nicotinic acid, it is likely that the bond is hydrolyzed and the nicotinic acid is made available. Quinolinic acid, a compound previously reported to be unable to replace niacin in staphylococci, and 3-pyridylsulfonic acid, isonicotinic hydrazide, compounds which were reported to be inhibitory to staphylococcal growth, also enhanced the growth and coenzyme contents at high concentrations. Some analogs such as nicotinamide-N-oxide and N-methylnicotinamide, were in fact

more effective in stimulating growth than niacin itself, and they yielded cells containing high concentrations of NAD(P).

f. Classification of niacin analogs. The various niacin analogs and related compounds can be classified into four groups based on their ability to replace niacin for staphylococcal growth, stimulation of the HMP pathway, and the NAD(P) concentrations of the cells (Table 9).

Group 1 niacin analogs (21) are able to replace niacin completely as a growth factor. There was maximal cell yield, NAD(P) content, and maximal stimulation of glucose oxidation via the HMP pathway when staphylococci were grown in media containing one of these analogs. It is interesting to note that most of the analogs are substituted in the 3-position.

Group 2 niacin analogs (11) are able to provide partial stimulation of growth, the stimulation of the HMP pathway, and of the NAD(P) content of staphylococcal cells. However, these analogs have to be added at high concentrations (500 µg /ml to the medium in order to stimulate growth. It can be seen that the analogs have substitutions at the 2, 3, 4, or 6 positions. 2-Chloronicotinic acid, 4-pyridylcarbinol, and 6-chloronicotinamide are included in this group.

Group 3 niacin analogs (9) have no effect on either the growth, the HMP pathway, or the NAD(P) content of staphylo-

Table 9

THE ABILITY OF VARIOUS PYRIDINE ANALOGS TO REPLACE NIACIN FOR STAPHYLOCOCCAL GROWTH, THE STIMULATION OF THE HMP PATHWAY, AND NAD CONCENTRATION

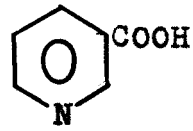
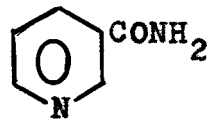
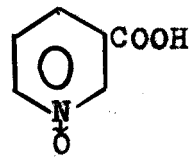
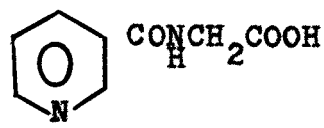
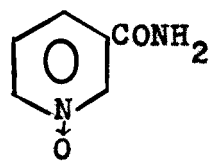
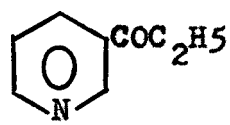
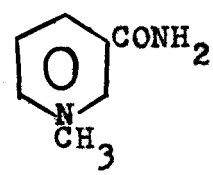
Compound	Structure	Stimulation Growth	HMP	NAD
Group 1				
Nicotinic acid (niacin)		++	++	++
Nicotinamide (niacinamide)		++	++	++
Nicotinic acid-N-oxide		++	++	++
Nicotinuric acid (nicotinyl glycine)		++	++	++
Nicotinamide-N-oxide		++	++	++
Ethylnicotinate		++	++	++
N-Methylnicotinamide		++	++	++

Table 9 (continued)

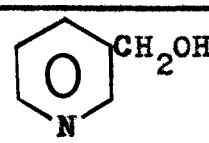
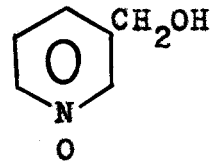
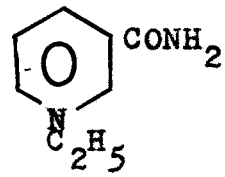
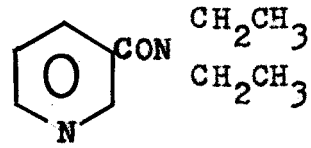
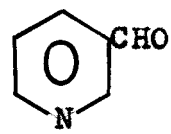
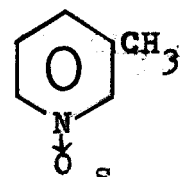
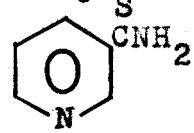
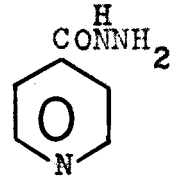
Compound	Structure	Growth	HMP	NAD
3-Pyridylcarbinol		++	++	++
3-Pyridylcarbinol-N-oxide		++	++	++
N-Ethylnicotinamide		++	++	++
Nicotinyldiethylamide (coramine)		++	++	++
Pyridine-3-aldehyde		++	++	++
3-Picoline-N-oxide		++	++	++
Thionicotinamide		++	++	++
Isoniazid (isonicotinylhydrazide)		++	++	++

Table 9 (continued)

Compound	Structure	Growth	HMP	NAD
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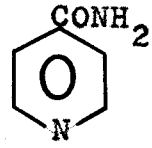
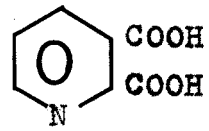
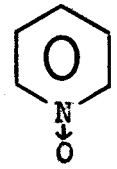
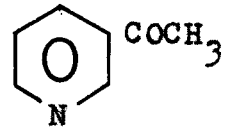
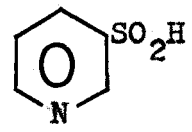
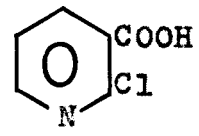
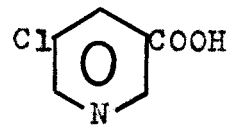
Isonicotinamide		++	++	++
Quinolinic acid		++	++	++
Pyridine-N-oxide		++	++	++
3-Acetylpyridine		++	++	++
Pyridine-3-sulfonic acid		++	++	++
Group 2 2-Chloronicotinic acid		++	++	++
5-Chloronicotinic acid		++	++	++

Table 9 (continued)

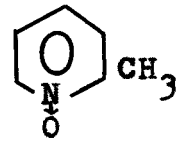
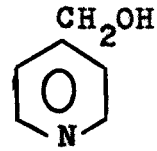
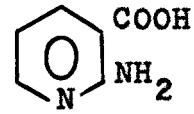
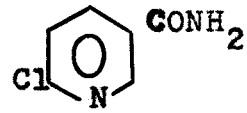
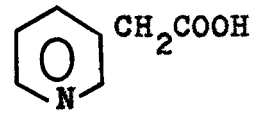
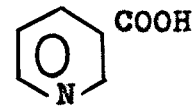
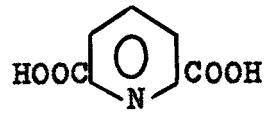
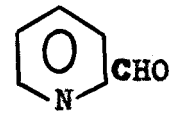
Compound	Structure	Growth	HMP	NAD
2-Picoline-N-oxide		+	+	-
4-Pyridylcarbinol		+	+	-
2-Aminonicotinic acid		+	+	-
6-Chloronicotinamide		+	+	-
3-Pyridylacetic acid		+	+	-
Picolinic acid		+	+	-
Dipicolinic acid		+	+	-
Pyridine-2-aldehyde		+	+	-

Table 9 (continued)

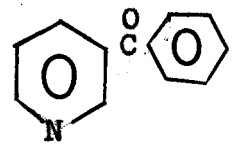
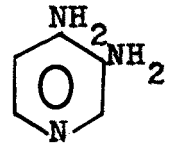
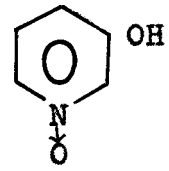
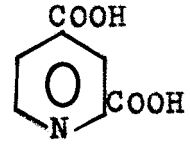
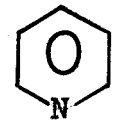
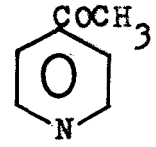
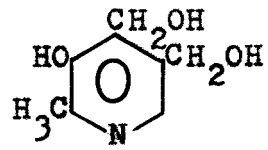
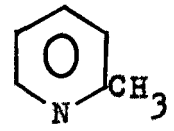
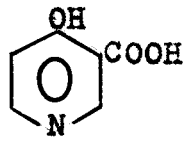
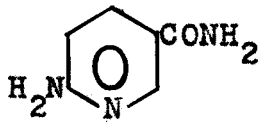
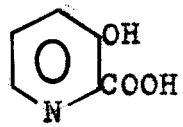
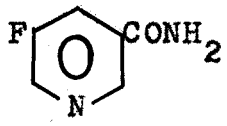
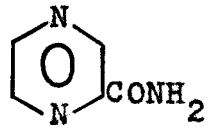
Compound	Structure	Growth	HMP	NAD
Group 3				
3-Benzoyl pyridine		-	-	-
3,4-Diaminopyridine		-	-	-
3-Hydroxypyridine-N-oxide		-	-	-
2,4-Pyridinedicarboxylic acid		-	-	-
Pyridine		-	-	-
4-Acetylpyridine		-	-	-
Pyridoxine		-	-	-
2-Picoline		-	-	-

Table 9 (continued)

Compound	Structure	Growth	HMP	NAD
4-Hydroxynicotinic acid		-	-	-
Group 4 6-Aminonicotinamide		i	ND	ND
3-Hyroxypicolinic acid		i	ND	ND
5-Fluoronicotinamide		i	ND	ND
Pyrizinamide		i	ND	ND

Symbols: ++, Maximal stimulation
+, Partial stimulation
-, No stimulation
i, Inhibition
ND, Not determined

cocci, even when added at high concentrations to the medium.

Group 4 analogs (4) completely inhibit the limited growth of staphylococci in the basal medium, when added in low concentrations. However, normal growth of staphylococci can resume when niacin is added along with the analogs in the medium. This group includes 6-aminonicotinamide, 3-hydropicolinic acid, 5-fluoronicotinamide, and pyrizinamide.

g. Effect of 6-aminonicotinamide on the concentration of NAD(P), AMP, ADP, glucose-6-phosphate, and 6-phosphogluconate.

Experiments were performed in order to determine if 6-aminonicotinamide (6AN), an inhibitor of staphylococcal growth, inhibited 6-phosphogluconate dehydrogenase as it does in certain animal tissues. When staphylococci were incubated in the presence of 6AN, the cellular NAD content decreases 74% after 3.5 hr, and by 84% after 6.5 hr of incubation (Table 10). Surprisingly, the NADP level remained relatively constant in spite of the drastic reduction of NAD. The decrease in NAD was observed to be more rapid as compared to cells incubated in the absence of 6AN (table 4). Although the ATP level remained relatively unchanged during the 6.5 hour incubation period, the AMP level was reduced almost 3-fold. The level of G6P was increased to 1.5 μmole/g. dry weight., and 6PG content was increased to 7.5 μmole after 6.5 hr of incubation.

Table 10

CHANGES IN NUCLEOTIDE AND SUBSTRATE LEVELS OF *S. AUREUS* INCUBATED IN THE PRESENCE OF 6-AMINONICOTINAMIDE

Cofactors and substrates	Nucleotide and substrate levels µmole/g. dry wt. +		
Hour	0	3.5	6.5
NAD	5.1	1.3	0.8
NADP	0.6	0.8	0.7
AMP	3.5	1.5	1.2
ADP	4.0	5.0	6.2
ATP	28.7	32.4	29.3
Glucose-6-phosphate	0.03	0.9	1.5
6-Phosphogluconate	0.02	2.4	7.3

+ Cells previously grown for 17 hr in 2% Vitamin-free Casitone medium supplemented with niacin (10 µg/ml), and thiamine (4 µg/ml), were washed and suspended in 0.05 M potassium phosphate buffer, pH 7.0, containing 1% glucose and 6AN (100 µg/ml). At intervals, samples were removed and extracted with phenol for nucleotide and substrate analysis. 6-Phosphogluconate was determined by the method of Hohorst (45).

This represents a dramatic increase from the initial barely measurable levels. However, the concentration of 6PG accumulated in the cells was 5 times greater than that of G6P. In a separate experiment, in conjunction with M. Wadke, we observed that the HMP pathway activity in staphylococci decreased rapidly when the cells were incubated in the presence of 6AN. Once again, the NAD(P) concentrations correlated with the activity of the HMP pathway. The inhibitory effect of 6AN on the HMP pathway, and the NAD level was also noticeable when staphylococcal cultures were grown with 6AN and low levels of niacin.

C. Uptake of niacin and its analogs by staphylococci.

a. Comparison of the uptake of ^{14}C-niacin and ^{14}C-quinolinic acid by S. aureus. Attempts were made to determine the reasons for the requirement of high concentrations of a niacin analog, such as quinolinic acid, for staphylococcal growth as compared to the low levels of niacin required for growth. The results of an experiment, comparing the uptake of ^{14}C-niacin, and ^{14}C-quinolinic acid, are shown in Fig. 5. There was a rapid rate of uptake of labelled niacin by the niacin-deficient staphylococci during the first few minutes of incubation, and it approached a saturation level by the end of 120 min, at which time almost half of the niacin administered had been incorporated into the cells. The amount of ^{14}C in the cells following admin-

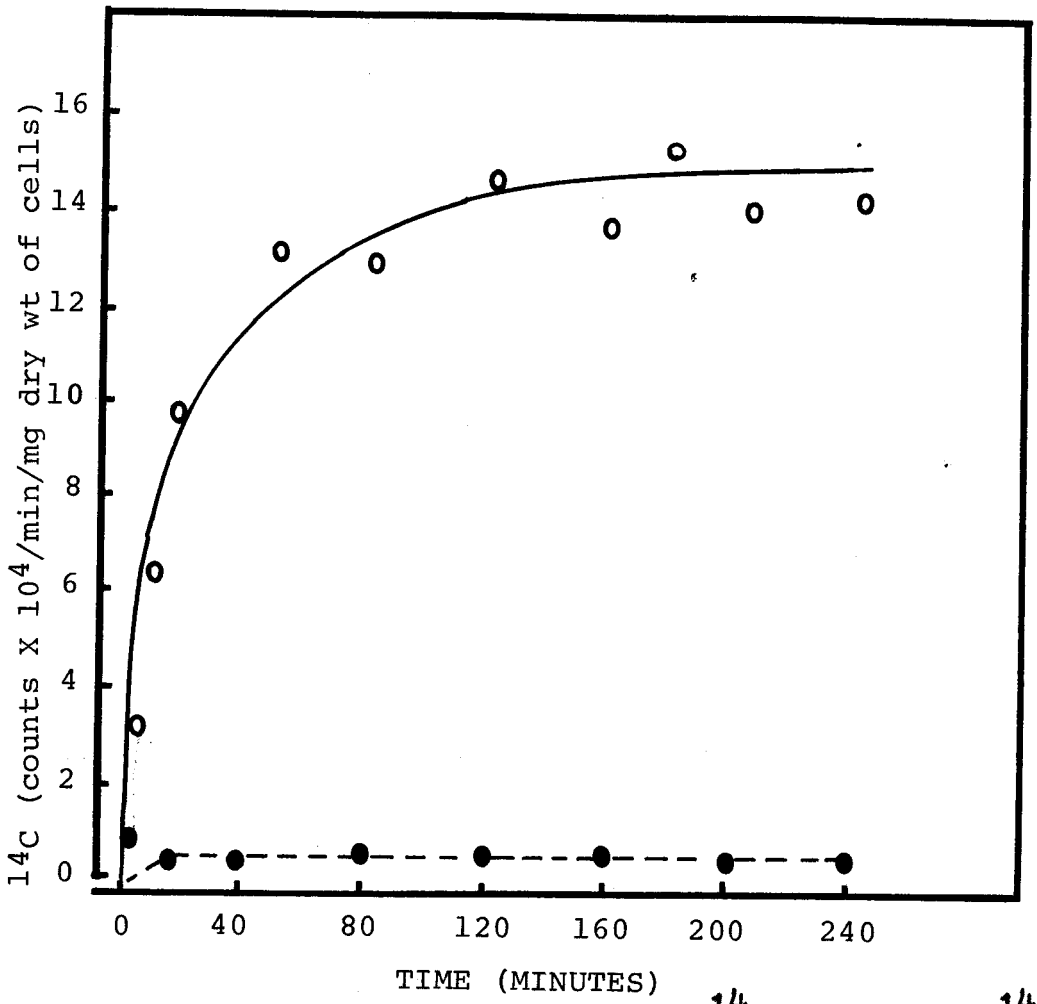


Figure 5. Comparison of the uptake of ^{14}C-niacin and ^{14}C-quinolinic acid by S. aureus.

After growth for 17 hr in 2% VFC medium supplewith thiamine (2 µg/ml), the cells were washed and suspended in 0.05 M K phosphate buffer, pH 7, containing 0.5 M glucose. One flask contained 2.4 µg/ml of ^{14}C-niacin (1 X 10^6 counts/min/ml medium), and the other 2.8 µg/ml ^{14}C-quinolinic acid (9 X 10^5 counts/min/ml medium). The flasks were incubated at 37 C on a rotary shaker and at intervals, 0.2 ml of the cell suspension was removed, filtered, and washed on a membrane filter, counted as described in Methods. o—o, ^{14}C-niacin; ●–●, ^{14}C-quinolinic acid.

istration of ^{14}C-quinolinic acid was very low, amounting to less than 1% of the total radioactivity of quinolinic acid administered.

b. The influence of temperature and incubation time on the uptake of labelled niacin by non-proliferating cells. The uptake of ^{14}C-niacin by a non-growing staphylococcal suspension was markedly influenced by the temperature, and the duration of incubation (Fig. 6). The uptake of labelled niacin by the viable cells suspended in buffer (with no glucose) increased linearly, and slowly with length of incubation time at 0 C. Of the total radioactivity administered, 8 % of the counts were taken up by the cells after 3 hr. In comparison, the uptake of niacin at 37 C was quite different and it had an initial rapid uptake (Fig. 5). The shape of the uptake curve at 37 C (Fig. 5) indicated that it involved an active transport system, while the results from the experiment at 0 C suggested that the uptake into the cells was by simple passive diffusion of niacin, independent of energy source. The linear increase was not due simply to an absorption phenomenon since heat-killed cells did not accumulate ^{14}C-niacin.

D. Isolation of nucleotides by anion exchange chromatography.

a. Anion exchange column chromatographic isolation of labelled intermediates from ^{14}C-niacin grown staphylococcal cells. When an extract, prepared from staphylococci grown in the

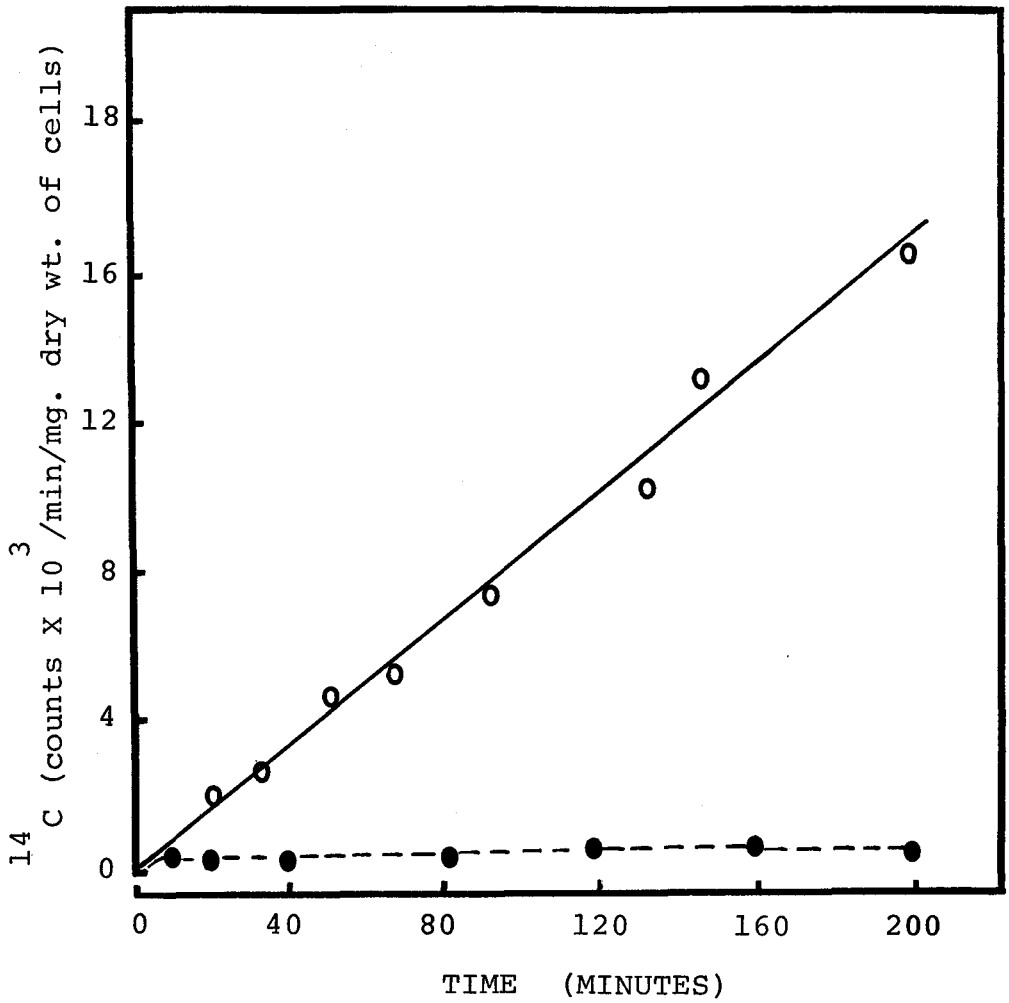


Figure 6. Uptake of ^{14}C-niacin by niacin-deficient washed-cell suspensions of S. aureus Towler at 0 C.

After growth for 17 hr in 2% Vitamin-free Casitone medium supplemented with thiamine (2 µg/ml), the cells were washed and suspended in 0.05 M K phosphate buffer, pH 7, containing 2 µg/ml of ^{14}C-niacin (1.78 X 10^{6} counts/min/ml), and incubated at 0 C. Heat killed cells were boiled for 2 min and used for the uptake studies. At various intervals, 0.2 ml of the cell suspension was removed, filtered, and washed on a membrane filter, and counted as described in Methods. o—o, viable cells; ●- -●, heat killed cells.

presence of thiamine, and labelled niacin, was chromatographed, a number of 260 nm radioactive absorbing peaks were detected. The pattern of elution from Dowex-1 (formate) column is shown in Fig. 7. At least five major radioactive peaks were eluted by this method. The radioactivity of the staphylococcal nucleotide extracts was almost entirely located in the NAD, NADP, and nicotinamide peaks. Although the major radioactive peaks have been characterized, the identity of many non-radioactive peaks, other than AMP and CMP, are unknown. Peaks III and IV from the column effluent were composed of two radioactive compounds which could be distinctly separated by paper chromatography, using a butanol-water solvent. The compound present in one of these peaks was exclusively nicotinamide. Following paper chromatography, peak VI appeared to be a mixture of NAD and CMP. During paper chromatography, the sample moved as two incompletely separated spots when chromatographed with an ethanol-ammonium acetate solvent. However, two distinct spots, identical in Rf values to NAD and CMP, were obtained when chromatographed in $MgSO_4$-phosphate-propanol solvent system. The radioactive spot fluoresced after treatment with methylethylketone and ammonia, and it was also examined spectrometrically from paper with water. In acid, the nucleotide exhibited an absorption maximum at 260 nm, and after 5 min in 1 M KCN, a

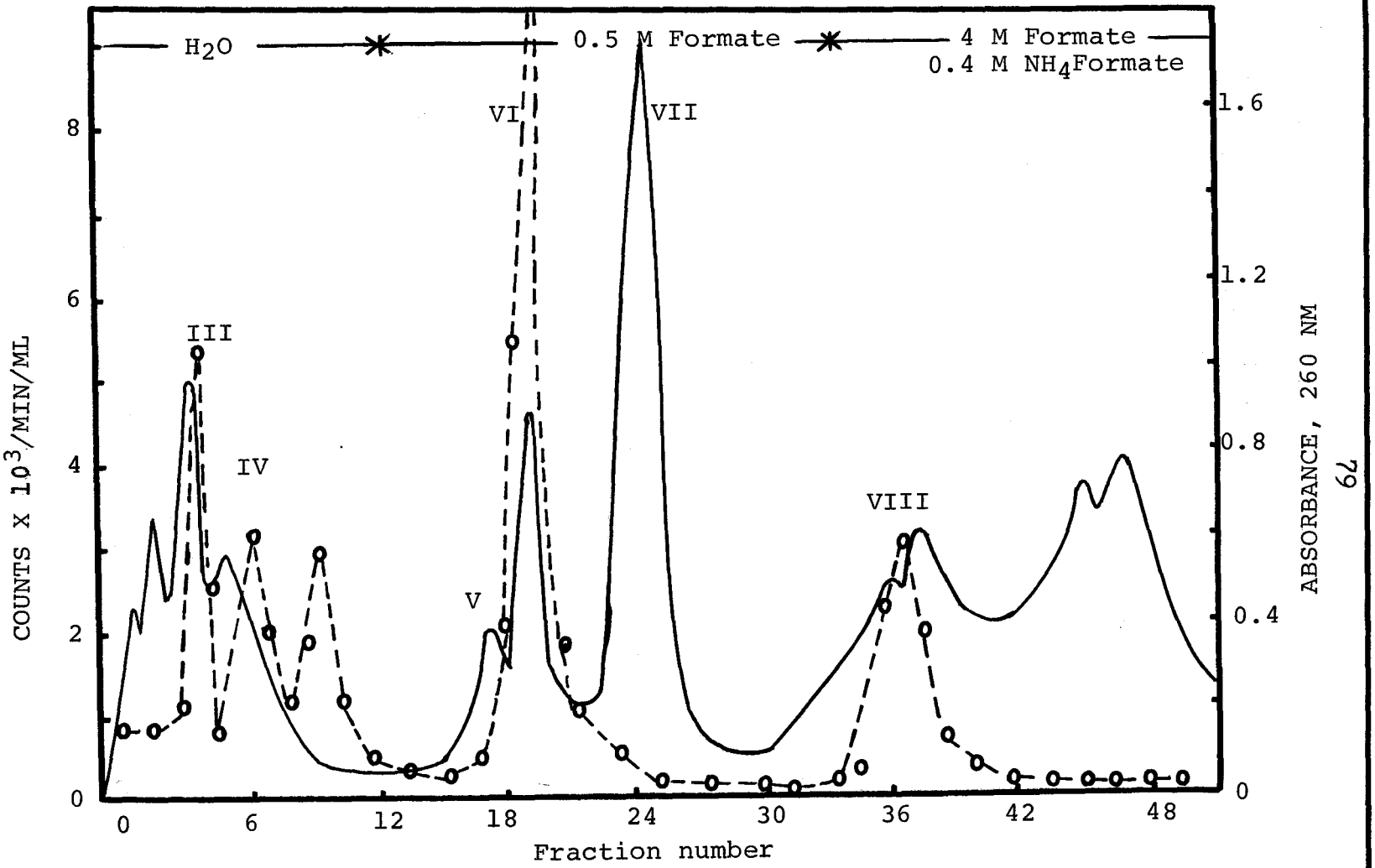


Fig. 7. Anion exchange chromatographic isolation of labelled intermediates from ^{14}C-niacin grown cells.

Staphylococcal cells were grown for 17 hr in VFC medium supplemented with ^{14}C-niacin (2×10^{-3} µg/ml), plus 2 µg/ml niacin, and thiamine (2 µg/ml).
—, absorbance; o–o, counts/min/ml.

second maximum developed at 325 nm. The nucleotide did not react with CNBr until hydrolyzed in 1 M NaOH. The addition of ethanol and alcohol dehydrogenase to a solution of radioactive sample caused an increase in extinction at 340 nm. The radioactive spot of peak VIII was broadly spread. However, when the radioactive spot was cochromatographed with authentic NADP, most of the radioactivity was found to correspond to the authentic NADP. It was also identified enzymatically as being NADP.

b. Anion exchange chromatographic isolation of labelled intermediates from ^{14}C-quinolinic acid grown staphylococci. In order to determine if growth with quinolinic acid yielded NAD, experiments were performed with ^{14}C-quinolinic acid. Radioactive nucleotides from the column elution of quinolinic acid grown cells were identified as described in Materials and Methods. The pattern of elution from the Dowex-1 (formate column is shown in Fig. 8. Fractions within peaks were pooled and lyophilized. Almost all of the radioactivity extracted from the cells accumulated in NAD. In peak I, no specific spot could be detected. In peak II, a complete spectrum analysis indicated the presence of adenine and cytidine derivatives; these were found to be NAD and CMP by paper chromatography in solvents 2 and 4. The adenine derivatives from the scan was found to be radioactive NAD.

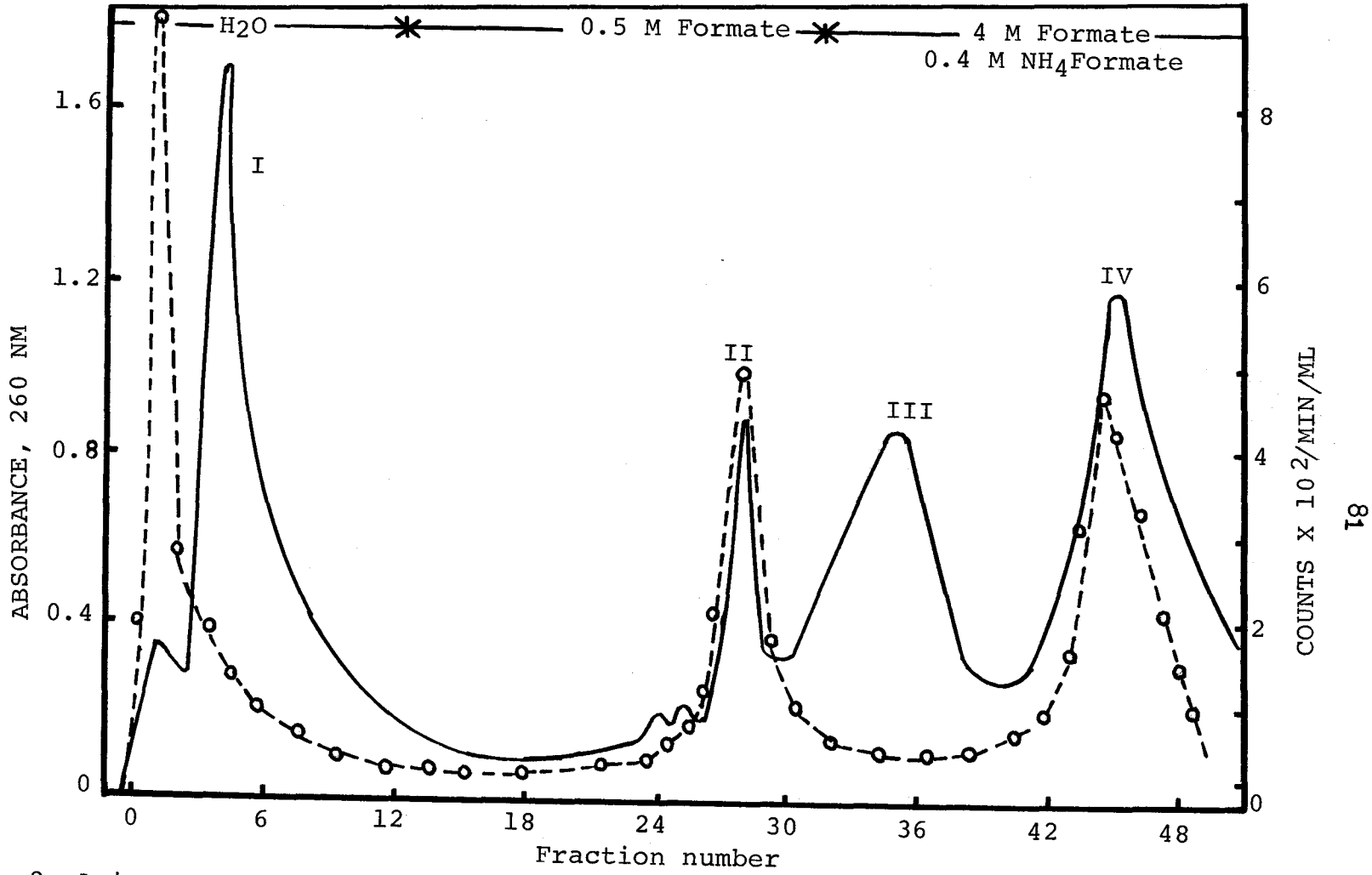


Fig. 8. Anion exchange chromatographic isolation of labelled intermediates from ^{14}C-quinolinic acid grown cells.

Staphylococcal cells were grown in VFC medium supplemented with 5 µCi (29.3 mCi/mM) quinolinic acid, plus 1.5 mg/ml quinolinic acid, and thiamine (4 µg/ml). ——, absorbance at 260 nm; o—o, counts/min/ml.

Peak IV consisted of a mixture of many nucleotides, and the chromatographic studies indicated that the predominant radioactive constituent was quinolinic acid. However, characterization was difficult because of the small amount of radioactive compounds available.

A similar experiment was done with cells grown in ^{14}C-isoniazid (carbonyl labelled). Even though there was a small uptake of radioactivity, no radioactive NAD or isoniazid analog could be detected from the chromatographic studies. This indicates that the group attached to C-4 does not end up as the carboxyl group of niacin.

c. Formation of ^{3}H- and ^{14}C-pyridine derivatives by staphylococcal cells from ^{3}H-pyridine-N-oxide and ^{14}C-niacin respectively. Fig. 9 shows the resolution of the absorbance at 260 nm, and the radioactivity of the various fractions of cell extract taken from a combination of two batches of cells. One batch of cells was grown in Vitamin-free Casitone medium, containing 2×10^{-2} µg/ml of ^{14}C-niacin (27.9 mCi/mM), and 2 µg/ml of niacin for 17 hr. Another batch of cells, previously grown in thiamine enriched phosphate buffer, pH 7, supplemented with 0.05 M glucose, and 12 µCi/ml of ^{3}H-pyridine-N-oxide (151 mCi/mM), and incubated for 8 hr at 37 C on a rotary shaker. At least three nucleotide peaks were eluted from the Dowex-1 (formate) column and each peak

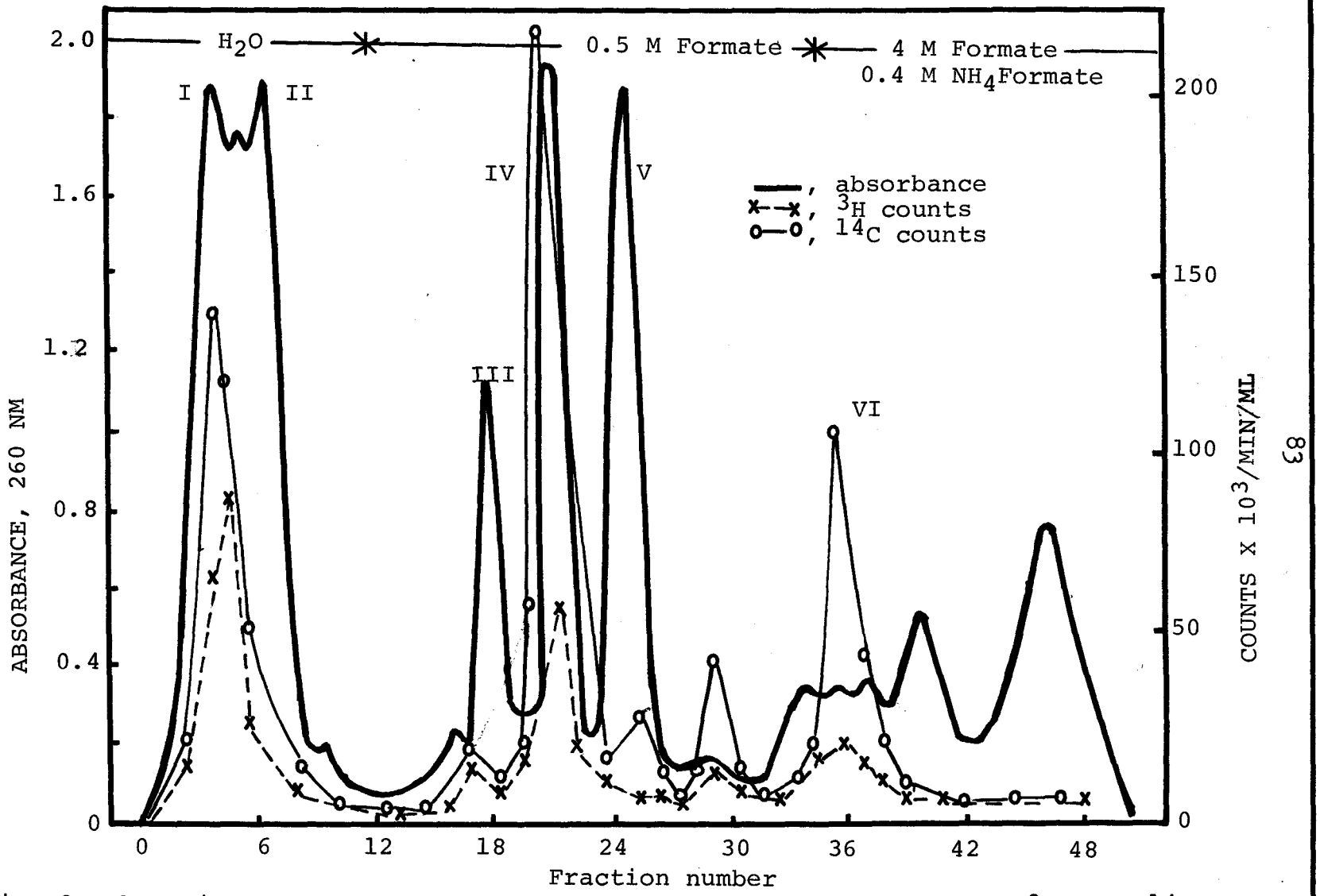


Fig. 9. The anion exchange column fractionation of nucleotides with ^{3}H- and ^{14}C-labels. The cell extracts were from 2 batches of cells; one batch grown in VFC supplemented with 2 X 10^{2} µg/ml niacin (27.9 mCi/mM), plus 2 µg/ml niacin and T (4 µg/ml); another batch of cells incubated in 1.25 mCi of pyridine-N-oxide (151mCi/mM) for 10 hr.

contained at least one radioactive compound. The ^{3}H-radioactive peaks correspond very closely to the ^{14}C radioactive peaks, with the exception of peak I and II. This elution pattern suggested that the nucleotides formed from the ^{3}H-pyridine-N-oxide were very similar to those obtained from ^{14}C-niacin. If this is so, the pyridine-N-oxide is converted to NAD. Most of the counts, both ^{3}H and ^{14}C, were localized in peak IV, which had been previously identified as NAD. Peak IV appeared from paper chromatography to be a mixture of NAD and CMP. However, when this fraction was chromatographed on ammonium sulfate-propanol-phosphate solvent, two distinct spots were obtained, and the NAD spot had a Rf of 0.23, and the CMP spot had a Rf value of 0.64. The radioactive spot fluoresced after treatment with methylethylketone and ammonia. The addition of alcohol dehydrogenase and ethanol to the radioactive spot eluted from the paper in a cuvette caused an increase in absorbancy at 340 nm. The increase at 340 nm could be removed with Neurospora NADase prior to the addition of alcohol dehydrogenase. The peak VI radioactive spot was tentatively identified as NADP. There was not enough material from this spot to characterize it further. When the radioactive spot was cochromatographed with normal NADP, it was found that most of the counts were localized in the NADP spot. Peak I and III were radioactive compounds eluted by water from the column.

DISCUSSION

The investigation of the role of adenine nucleotides in the regulation of glucose oxidation through the Embden-Meyerhof and/or hexosemonophosphate pathway, as well as the tricarboxylic acid cycle, is a complex process and many factors are involved. These include the relative amounts, and the activities of the various enzymes of the glycolytic pathways, the concentrations of the cofactors, such as NAD, NADH, NADP, AMP, ADP, GTP, ATP, acetylCoA, and the concentrations of other metabolites that may interact with, or compete with, glucose and its intermediate. One of the most important factors appears to be the nicotinamide nucleotides, which play a central role in the oxidation reactions of all organisms, and the intracellular redox potential of these compounds is an important parameter in the regulation of cell metabolism. In general, the knowledge of the free nucleotide patterns in the organisms could provide an index as to the nature and extent of the predominant metabolic processes occurring within the cells.

Knight (63,64) first observed that niacin was an essential vitamin required for the growth of staphylococci. The only known physiological role of niacin is to act as precursor for the biosynthesis of NAD and NADP. Kligler et al. (61) first reported that niacin was involved in staphylococcal glycolysis while thiamine was needed for the further oxidation of pyruvate formed in glycolysis. Later, Hughes (47) postulated

that niacin stimulated glycolysis by being converted into the nicotinamide coenzymes, NAD and NADP.

We previously found that the proportion of glucose metabolized via the HMP pathway by resting cell suspensions of staphylococci was stimulated 2- to 3-fold when niacin was added to a Vitamin-free Casitone growth medium. Concomitantly, there was a 10- to 15-fold increase in the NAD level, and a 2- to 3-fold increase in the NADP level. Similiar increases in the levels of these coenzymes occurred whether the cultures were grown in the presence or absence of thiamine, as long as niacin was present in the medium.

There are a number of reports which have indicated the relative degree of the operation of the HMP pathway to be governed by the activity of glucose-6-phosphate dehydrogenase (G6PD). For example, Osmond and Ap Rees (96) found the HMP pathway in yeast to be controlled by the variation in the amounts of the enzyme, G6PD, present in the cells. In S. aureus, the G6PD and 6PD were found to be NADP-linked (46), confirming the earlier report of Strasters and Winkler (115). In the present studies, there was about 2- to 3-fold increase in the activity of these enzymes and also a concomitant 3-fold increase in the operation of the HMP pathway when the growth medium was supplemented with niacin. These observations seem to support the suggestion that the level of G6PD is limiting the operation

of the HMP pathway and that the increased amount of G6PD and 6PGD might increase the amounts of glucose to be oxidized by the HMP pathway. That this was not the correct answer for staphylococci became apparent when we found that the operation of the HMP pathway could also be stimulated by the addition of niacin without increasing the amount of G6PD in the cells.

In order to observe whether the increased operation of the HMP pathway required increased enzyme synthesis in the presence of vitamins, washed cells were incubated in buffered glucose without a nitrogen source (resting cells), and in the presence of a number of antibiotics known to be inhibitors of protein synthesis. Thus, when we incubated washed suspension of niacin-deficient staphylococci with niacin in the presence of chloramphenicol, puromycin, and actinomycin D, we found (Table 5) that the antibiotics did not prevent the stimulation of glucose oxidation via the HMP pathway, and the stimulation in the NAD(P) contents by niacin under conditions which precluded protein synthesis. In fact, the antibiotics, actinomycin D, when added along with niacin during incubation, stimulated the operation of the HMP pathway to a greater extent than in the controls of staphylococcal suspensions without antibiotic.

There was no increase in the amounts of NADP-linked enzymes that were measured, even in the presence of chloramphenicol, such as the HMP enzymes, G6PD, and the TCA cycle enzyme, ICD.

In all cases, the HMP pathway stimulation was always accompanied by the increase in the total NAD(P) content but with or without changing the amount of G6PD in the cells. Furthermore, we observed that when staphylococci were grown in a synthetic medium containing niacin, the operation of the HMP pathway was much higher even though the amount of G6PD was 3- to 4-fold lower than the cells grown in Vitamin-free Casitone under similar conditions. Therefore, there was no direct correlation between the level of G6PD, and the activity of the HMP pathway. The increased enzyme levels of G6PD and other enzymes found after growth in niacin supplemented media appeared to be secondary to the increased levels of nicotinamide coenzymes in staphylococci. Evidently, the activities of G6PD and transketolase could only play a secondary role in determining the capacity of the HMP pathway in staphylococci. Although the induction and repression of enzyme synthesis can be considered to be an alternate control in cellular regulation of metabolism, it is clear that the vitamin, niacin, is acting at a different level in staphylococci, perhaps as a fine control for the utilization of the EM and HMP pathway, through the regulation of the levels of NAD and NADP.

Recent reports by Srere (113) suggest that enzymes are actually present in tissues in greater concentration than the normally measurable values. He observed that the apparent

concentration of a number of enzymes was actually five times greater than the calculated cellular concentration, because the enzymatic assays for the enzymes in vitro, which are performed with low concentrations of enzymes in an extract media, failed to measure the true total intracellular concentration of most enzymes within the cells. Since the intracellular enzymes are present in excess of the amount necessary for normal cell growth, then any increase in the availability of the substrates, either glucose-6-phosphate, or cofactor, NAD(P), might be responsible for the stimulation of the HMP pathway. Glucose-6-phosphate is likely to exert a limiting role in the operation of the HMP pathway since it can be derived from wider sources of metabolic intermediates, whereas NAD(P) are derived only from their precursor, the vitamin niacin.

The effect of ATP concentration on the glycolytic pathways has been previously reported by other workers. For instance, Tsuboi et al. (121,122) demonstrated that the increase in ATP level was followed by the increase in the activity of the glycolytic pathways even under conditions in which the NAD level was decreased. However, one must be careful to note that this occurred in mammalian erythrocytes which lack a functional TCA cycle, and in which the energy supply is dependent solely on ATP production from the EM pathway. In staphylococci, the energy requirement can probably be satisfied by

the oxidation of amino acids via mechanisms which involve the TCA cycle. The role of adenine nucleotides, such as AMP, ADP, and ATP, in the regulation of the glycolytic pathway and coenzyme synthesis could not be discerned from our experiments. There was no significant correlation between the levels of NAD(P), which correlated well with the degree of glucose oxidation via the HMP pathway, and the adenine nucleotide levels. In general, staphylococci that had low HMP and TCA cycle activities when grown in the absence of niacin and thiamine had ATP/AMP ratios close to 1 (Table 3). The addition of niacin to the medium raised the ATP/AMP ratios to 5 and above. But the ATP content was highest when both niacin and thiamine were added as supplement to the growth media. This is understandable since the presence of both vitamins, the TCA cycle operated maximally and supplied all the energy requirement.

The involvement of ATP in NAD synthesis is well understood and 3 moles of ATP are required for the synthesis of 1 mole of NAD. Since this also represents considerable expenditure of energy for the microorganisms, it might be expected that ATP is involved in the regulation of NAD biosynthesis. Indeed, Nakamura et al. (87), using a purified beef liver extract system, demonstrated that the NAD biosynthesis was regulated by ATP. In the absence of ATP, deamidonicotinic mononucleotide was

converted to niacin ribonucleotide and eventually to niacin whereas when ATP was available, no niacin accumulated and NAD formation occurred. In staphylococci, the ATP was found in much higher concentrations when the total NAD(P) content in the cell. The total NAD(P) content increased to a higher level in staphylococci upon the addition of niacin alone to the growth medium, and under conditions where the TCA activity was not stimulated and the cellular ATP level was not at a maximum. Incubation of resting cell suspensions of staphylococci resulted in a drastic decrease in total NAD(P) level of 50% whereas the ATP level was simultaneously increased by 100% by 3 hr of incubation (Table 4). Moreover, it is generally believed that cells usually produce sufficient ATP, or even overproduced it to fill the physiological needs of the cells (65). For instance, human erythrocytes contain an average concentration of 1 μM of free adenine nucleotide/ml of packed cells and most of this is ATP.

There are many reports which state that the HMP activity is rate-limited by the supply of NADP to the NADP-linked G6PD. For instance, Takebe and Kitahara (118) found that in the lactic heterofermentors, the ratio of NADP/NAD was 0.11-0.29, as compared to the ratio of NADP/NAD of 0.02-0.05 in homofermentors. Thus, the heterofermentors had a higher level of NADP and a more active HMP pathway than the homofermentors.

Eagon(28) noted that in Pseudomonas natriegens, the operation of the HMP, and also the TCA cycle, were both rate-limited by the supply of NADP to the NADP-linked G6PD and ICD respectively. Wenner et al. (126) indicated that the limiting factor in the operation of the HMP pathway in ascites tumor cells was dependent on the availability of the electron transport system to reoxidize NADPH to NADP. This was supported by the fact that the addition of artificial electron acceptors, such as methylene blue, and phenazine methosulfate, stimulated carbon-1 oxidation and hence increased the operation of the HMP pathway. Based on these previous reports, we initially assumed that the 2- to 3-fold increase in NADP in staphylococci, although much smaller than the 15-fold increase in NAD level, was directly responsible for the increased HMP pathway, and that the NAD level was unimportant. However, we found that the HMP pathway activity could also be rate-limited by the availability of NAD in the cell under certain conditions.

The involvement of the transhydrogenase system (TD) in stimulating the operation of the HMP pathway in bacteria had been reported. Eagon (27,28) found a strong correlation between the simultaneous presence of the readily demonstrable NADPH-oxidase and the transhydrogenase in those bacteria that predominantly utilized the HMP and/or ED pathways for glucose oxidation. The transhydrogenase catalyzes the following reaction.

$$NADPH + NAD \longleftrightarrow NADP + NADH$$

From our data, we observe that the relative concentration of NAD in the cell could affect the operation of the HMP pathway. When the NAD level in staphylococci decreased below 2.0 μmole/g. dry wt., the operation of the HMP pathway was also decreased even though the NADP content remained constant. This seems to suggest that the transhydrogenase system which requires NAD as a substrate is important and involved in replenishing the NADP supply through the TD system by reaction with the NADPH produced from the HMP pathway and TCA cycle. Besides, the NADH produced could then serve as a energy source in the cell metabolism. A slight increase in the transhydrogenase activity in staphylococci was observed (46) when the HMP pathway was stimulated by niacin. Our preliminary investigation indicated that the HMP pathway of resting cell suspensions of staphylococci could also be stimulated by electron acceptors such as pyocyanin, even without changing the level of NAD(P) in the cells.

We observed that the total NAD(P) content in staphylococci could be varied either by growing the cells in media containing suboptimal amounts of niacin or by "starvation" of niacin-sufficient cells by incubation in buffered glucose solutions. Thus, incubation of washed-cell suspensions of staphylococci, previously grown in a niacin-enriched medium, led to a decrease in NAD content, but the NADP content remained unchanged, or even slightly increased (Fig. 1 and Table 4). Although there

was a decrease in NAD from 6.7 to 2.5 μmole/g. dry wt. (Table 4), this drop in the level by more than half in the course of 6 hours of incubation did not result in a decrease in the operation of the HMP pathway. This is attributed to the fact that the total cellular NAD(P) content in staphylococci during this time of incubation still remained high enough to provide maximal operation of the HMP pathway, owing to the initial high content of NAD(P) in the cells. Therefore, the coenzymes were actually overproduced in staphylococci as far as their physiological needs for glycolysis were concerned. These results also confirm and extend the findings of Nozawa et al. (92) who observed the loss of a large amount of NAD, but not NADP following the starvation of E. coli cells.

In order to begin the starvation of the staphylococci with initial lower NAD(P) levels, staphylococci were grown in Vitamin-free Casitone medium supplemented with 0.5 μg/ml of niacin. The results in Fig. 1 showed that as the NAD content dropped below 2.2 μmole even under conditions in which the NADP level was increased slightly, there was a concomitant decrease in the HMP pathway in the cells. These results strongly indicated that the operation of the HMP pathway in staphylococci tended to follow the changes of total NAD(P), and not the NADP level alone. To our knowledge, this is the first instance in which the NAD content of the living cells has been

shown experimentally to be involved in controlling the reactions of the HMP pathway. To reinforce this finding further, restoration of the HMP pathway to maximal operation could be brought about in the starved cells by elevation of the total NAD(P) content of staphylococci. This was done by the addition of niacin to the incubation medium after the time when the coenzyme level had reached a critical low level. A 2-fold increase in NAD content was enough to restore the activity of the HMP pathway to its original high level.

The increased activity of the HMP pathway following addition of niacin, with no added thiamine in the medium eliminates the possibility that the level of thiamine is responsible for limiting the amount of glucose oxidized by the HMP pathway. On the other hand, thiamine, in the form of DPT, apparently exerted an important regulatory role on the activity of the TCA cycle. Staphylococci accumulated 10-fold increased amounts of pyruvate when thiamine was ommitted from the medium; oxidation of the cellular pyruvate occurred immediately following the addition of thiamine. In staphylococci, the TCA cycle activity remained low, even in the presence of increased NAD(P) levels, unless thiamine was present in sufficient amounts in the growth medium. This indicates that the TCA cycle activity is not solely dependent on the total NAD(P) content. This would suggest that at least two regulatory sites are involved in the

TCA cycle activity in staphylococci. One site is between pyruvate oxidation to citrate, a location where DPT coenzyme is required as a cofactor for pyruvate decarboxylase, and the other site may be the NADP-linked ICD. This is supported by the fact that maximal operation of the TCA cycle occurred only when both niacin and thiamine were present in the growth medium. The stimulation of the TCA cycle activity by thiamine is staphylococci is not a coenzyme induced synthesis of the TCA cycle enzymes since the TCA cycle activity could be restored under conditions in which enzyme synthesis was prevented by antibiotics (Table 5).

An important observation made during the course of this study is that there were two different niacin concentrations needed by staphylococci; one level for maximal growth, and a higher level for the maximal NAD(P) levels, and the HMP pathway activity. Although no significant increase in the total NAD(P) content or HMP pathway activity could be detected when the growth medium was supplemented with niacin in concentrations below 0.1 μg/ml (1.5×10^{-5} M), there still was a maximal rate of staphylococci grown in suboptimal level of niacin was independent of the increased operation of the HMP pathway to provide the intermediates for cell multiplication. In order to obtain maximal levels of NAD(P), and the operation of the HMP pathway, at least 1 μg/ml (1.5×10^{-4} M) was required. It

appeared that a 5-8% consumption of glucose via the HMP pathway was sufficient to provide maximal rate of growth of staphylococci (Fig. 4). It has been shown in yeast that 1% of glucose consumed was metabolized via the HMP pathway, and converted to ribose-5-phosphate, which was subsequently found to be incorporated into the free nucleotides and nucleic acid.

In the present studies, when the concentration of niacin in the staphylococcal growth media was increased above 0.1 µg /ml, there were increasing amounts of NAD formed until a concentration of 1 µg/ml was reached, at which time no further increase in total NAD(P) was noticeable. Staphylococci grown in the presence of 0.25 µg/ml of niacin had only 33% of the NAD content found in cells grown in optimal amounts of niacin. If we take 1 g of dry cells, corresponding to a volume of 2.15 ml of fresh cells (118), the intracellular concentration of NAD and NADP in staphylococci can be calculated to be 2.2×10^{-3} M, and 2×10^{-4} M respectively, using an cellular NAD content of 5.5 µmole, and NADP content of 0.5 µmole/g. dry wt. This unexpectively high calculated value for the cellular NAD content suggested that in staphylococci, the NAD coenzyme is present in excess of the amount necessary for the maximal operation of the glycolytic system. It has been estimated that the glycolytic dehydrogenases are present at a concentration in the order of 10^{-5} M (118). Even if we assume

that 3 or 4 moles of NAD are needed per mole of enzyme, there still is a great excess of NAD. Our data also indicate that the NAD/NADP ratio was always greater than 10 when the cells were grown with optimal amounts of niacin in the growth medium. Imsande (52) found that the ratio of NAD to NADP concentration equalled about 10:1 in E. coli. However, in the present work, the ratio of NAD/NADP could be varied markedly depending upon whether the cultures were grown with low (less than 0.5 μg/ml), or high (greater than 0.5 μg/ml) concentration of niacin. For the stimulation of the HMP pathway in staphylococci, a 2-fold increase in NAD and NADP content, to levels of about 1 μmole and 0.5 μmole/g. dry wt., respectively, was sufficient for the process.

We could not detect any significant amounts of reduced coenzymes, NADH and NADPH, in the staphylococci when they were grown in media supplemented with niacin and/or thiamine. Consequently, the role of the reduced NADH and NADPH on the regulation of glucose catabolic pathways could not be discerned from our experiments. However, Glock et al. (34) had reported that the decrease in the total pyridine nucleotides was reflected in the decrease in both the oxidized as well as the reduced nucleotides and vice versa.

During the early stages of the studies just described, attempts were made to selectively inhibit either NAD or NADP

levels by adding a number of known analogs of niacin that were known to be inhibitors in animal tissues. To our surprise, we found that compounds such as 3-acetylpyridine and pyridylsulfonic acid, could completely replace niacin as the required vitamin for the growth of staphylococci. Consequently, this finding was pursued to determine the extent staphylococci could use these pyridine derivatives and to ascertain whether or not the compounds were indeed being converted to NAD and NADP or whether the analogs of NAD or NADP were being formed.

We found that a number of 1-, 2-, 3- or 4-substituted pyridine compounds, including pyridyl-N-oxide, N-methylpyridine, 2-, or 3-methylpyridine, pyridylcarbinol, pyridylaldehyde, and isonicotinic acid or hydrazide, could replace niacin as a growth factor when the analogs were added, usually at high concentrations, to the growth media in the presence of thiamine (Table 7 and 9). Quinolinic acid, previously reported to be ineffective as a niacin replacement, and both isoniazid and pyridylsulfonic acid, which were reported to be inhibitory for staphylococci, were also found to stimulate staphylococcal growth when they were added at higher concentrations than those used in the original studies. Of the more than 40 compounds tested, only pyridine, 2- or 3-hydroxypyridine, 5-fluoronicotinic acid, and 6-substituted pyridines,were found to have no stimulatory effect on growth. One compound in this

latter group, 6-aminonicotinamide, was observed to be inhibitory for the growth of staphylococci. The various N-oxides of pyridine compounds, especially nicotinic acid-N-oxide, and nicotinamide-N-oxide, appeared to be better growth factors than niacin. For example, the maximal growth of staphylococci using nicotinamide-N-oxide was 370 Klett units while the maximal value with niacin was 340 Klett units (Fig. 3). The ability of staphylococci to grow normally in concentrations of these niacin analogs that were 5,000 to 20,000 times greater than the concentration of niacin was surprising. This indicated that the niacin analogs were relatively non-toxic to staphylococci, even when present in high concentrations. On the contrary, high concentrations of nicotinamide was observed to lower the endogenous respiration of rat liver homogenates and yeast, but the inhibition could be reversed by the addition of NAD. Many niacin analogs could substitute for niacin as a growth factor if higher concentrations of the analogs were employed. McIlwain (82) noted that all of the compounds which failed to permit staphylococcal growth, were not toxic to the bacteria since growth ensued on subsequent addition of nicotinamide. He observed that quinolinic acid, trigonelline, and nicotinyl diethylamide (coramine), did not stimulate growth during the first 24 hr, but later served as a replacement for niacin. However, pyridylsulfonic acid and its

amide inhibited staphylococcal growth throughout the observation period. The failure of these compounds to stimulate growth was attributed to their non-utilizability. Landy (74) postulated that reduction of ethylnicotinamide was brought about by the introduction of the ethyl group, or any addition to the side chain. In general, all the early workers stressed the great structural specificity of niacin as a growth requirement for staphylococci. Many niacin analogs which had been previously reported to be inactive, such as nicotinyl-N-diethylamide, 3-acetylpyridine, thionicotinamide, isoniazid, pyridylsulfonic acid, and quinolinic acid, were able to substitute for niacin as a growth factor (Table 10). In contrast to the report of Mc Ilwain (82), who indicated that pyridylsulfonic acid (PSA) and its amide were inhibitory to the growth of various bacteria, we observed that PSA could actually stimulate the growth, and the coenzyme content in staphylococci. The basic difference in our observation and the early workers was perhaps the concentration of niacin analogs and the basic media used for testing. Earlier workers used concentrations of the analogs comparable to, or slightly higher than, the concentration of niacin required. We also found that most of the analogs were inactive as niacin replacements when they were used at concentrations equivalent to the growth stimulatory concentration of niacin. Cote and Oleson

(20) used concentrations of niacin analogs in the range of 0.12 to 0.5 μg/ml to test the activity of 22 N-substituted nicotinamide derivatives toward Lactobacillus plantarum. Pelczar and Porter (98) added 2 X 10^{-5} M (ca. 3 μg/ml) of related pyridine compounds to the culture medium to test the utilization of the various analogs by the various species which required niacin for growth. They found that picolinic acid, quinolinic acid, pyridine-betaine carboxylic acid and trigonelline were inactive, but that compounds that were biologically active for S. aureus were also active for the majority of the Proteus strains.

Ellinger et al. (30) in their comparative survey of the biological activities of the various compounds related to niacin, found that all of the compounds which could be utilized by the bacteria, were also utilized by the higher species, rat. All three bacteria examined by them utilized niacin, niacinamide quinolinic acid, and β-picoline, although the latter two compounds were much less effective than niacin. In the present study, staphylococci were found to use quinolinic acid as a replacement for niacin. The ability to utilize quinolinic acid, however, might indicate that the quinolinic acid-tryptophan pathway for niacin synthesis might be present in staphylococci. This seems unlikely, however, when we consider the wide variety of the pyridine compounds that can also replace

niacin. Growth stimulation, and increase in coenzyme content occurred only when quinolinic acid was added to the medium at concentrations of not less than 500 μg/ml. The necessity of using such a high concentration of quinolinic acid might possibility be due to the problem of uptake of quinolinic acid by staphylococci. Radioactive uptake studies using labelled niacin analogs, ^{14}C-quinolinic acid, ^{14}C-isoniazid, and ^{3}H-pyridine-N-oxide, indicated that the analogs were taken up by the cells only to a small extent (Fig. 5). Our studies with labelled quinolinic acid indicated radioactive NAD was actually formed from labelled quinolinic acid. Since a number of pathways for niacin biosynthesis are present in bacteria, it is possible that staphylococci might possess alternate pathways for niacin biosynthesis. In E. coli, the de novo synthesis of NAD occurs through the synthesis of quinolinic acid, from aspartate and a 3-carbon compound, and its subsequent conversion into NAD without the occurrence of free niacin as an actual intermediate (2). In addition to the de novo pathway, E. coli can also utilize niacin and niacinamide for NAD synthesis by employing a salvage pathway (101). The pathway for the conversion of quinolinic acid into NAD is present in a number of bacteria and in mammals.

Isoniazid, a potent antituberculous drug, was found to be effective in stimulating staphylococcal growth when it was

added at concentrations greater than 500 μg/ml (Table 8). Increased levels of NAD(P), paralleled with the increased operation of the HMP pathway, occurred when this analog replaced niacin in the medium. These results in which isoniazid was able to stimulate staphylococcal growth was quite different than those with mycobacteria which are inhibited by this analog. The NAD in staphylococci contained no radioactivity when they were either grown or incubated in the presence of ^{14}C-carbonyl labelled isoniazid. These results further suggest that the isoniazid analog of NAD was not formed from isoniazid. The stimulation of growth and coenzyme content could be the result of the conversion of isoniazid into cellular NAD(P) by mechanism not yet known. Furthermore, it indicates that the C at the 4 position of the pyridine ring does not become the carboxyl group on the 3-position of niacin.

Recent reports (10,128) have indicated that the primary inhibitory effect of isoniazid in mycobacteria was to cause a decrease in NAD content in the cells as a result of the indirect activation of NAD glycohydrolase which mediates the breakdown of NAD. There is as yet no report suggesting that the de novo synthesis of isoniazid coenzyme occurs in living cells. However, Anderson et al. (3), and Kaplan et al. (57) were able to demonstrate the in vitro formation of isoniazid-NAD using the pig NADase system.

We observed that the NADase activity in S. aureus could not be detected in the cell-free extracts of the sonicated cells. The enzymatic breakdown of NAD occurred only after the whole sonicated cell extract was boiled for 2 min, as previously reported by others (117). Apparently, this is due to the destruction of heat-labile inhibitor of NADase. Preliminary experiments in the present study indicated that there was no difference in the rate of breakdown of NAD between staphylococci grown with or without vitamins in the media, thus eliminating the possibility that NADase activity was involved in the utilization of isoniazid.

6-Aminonicotinamide (6AN) has been known to be a potent nicotinamide antagonist in animals. In the present study, it was found that incubation of staphylococci in the presence of 6AN resulted in a rapid decrease in the NAD content (Table 10). Furthermore, there are reports (25,44) which indicated that 6AN coenzymes were formed after administration of 6AN, but this occurred in the mammalian systems. We also observed the increase in the intracellular glucose-6-phosphate and 6-phosphogluconate contents of staphylococci following exposure to this analog, just as had been reported in mammalian systems by Herken and Lange (44). The large increase in the 6-phosphogluconate and the lesser increase in glucose-6-phosphate, become very prominent when the NAD level in staphylococci

was limiting. When there was an excess of NAD in the cells, no significant increase in G6P and 6PG could be detected. The increase in the substrates and the decrease in the total amount of NAD(P) was accompanied by the decrease in the operation of the HMP pathway (44). However, it cannot be determined on the basis of this investigation, whether the metabolic change in the staphylococci was due to the formation of 6AN-NAD or due to the direct inhibition of 6AN itself on 6PGD.

Pyridine derivatives which were substituted in other than the β-position were found to be rather ineffective in stimulating growth and increased NAD(P) content. The 3-substituted pyridine compounds were the most effective ones for replacing niacin as a growth factor. 2-Chloronicotinamide, 6-chloronicotinamide, and 2-picoline were quite effective in stimulating growth but increased levels of NAD(P) were harder to detect. We have no explanation as to why the hydroxylated pyridine compounds, such as 6-hydroxynicotinic acid and 3-hydroxypyridine, were completely inactive. In fact, of all the 3-substituted pyridine derivatives tested, only 3-hydroxypyridine was found to be inactive.

Preliminary experiments by Wadke (personal communication) indicated that 6-hydroxynicotinic acid stimulated the TCA cycle activity of staphylococci. This thiamine-like effect was noticeable only when the cells were previously grown in the

presence of the analog, and the washed-cell suspension was subsequently incubated in the presence of niacin. Harary (43) noted that under anaerobic conditions, Pseudomonas fluorescens could catalyze the reversible oxidation of nicotinic acid to 6-hydroxynicotinic acid, and its oxidation of nicotinic acid to 6-hydroxynicotinic acid, and its subsequent conversion to propionic acid, acetic acid and ammonia. However, no explanation of the thiamine-like effect of 6-hydroxynicotinic acid is apparent.

5-Fluoronicotinamide was found to be inhibitory to staphylococci in the present study. Hughes (47) postulated that growth inhibition by this compound was caused by the inhibition of NAD synthesis. He found S. aureus to be inhibited by the 5-halogenated niacin analogs in the following order of effectiveness: 5-fluoro>chloro>5-bromo>2- and 6-fluoronicotinic acid. However, evidence that the fluoro analog may be easily metabolized by bacteria and that the C-F bond remained intact is provided by studies on the oxidation of fluoronicotinic acid.

When the N-oxide derivatives of pyridine, such as nicotinamide-N-oxide, nicotinic acid-N-oxide, and pyridine-N-oxide, replaced niacin in the growth medium, higher yields of cells were obtained than when niacin was added. The N-oxides have been reported to be a group of highly reactive compounds and biological reduction of the N-oxides into parent compounds can

occur. When the N-oxides were added to the growth medium, they resulted in an increased NAD(P) content in staphylococci (Table 8). Thus, the N-oxides of pyridines which are stimulatory to staphylococci were most likely being reduced and converted into niacin. In fact nicotinamide-N-oxide is one of the common excretory products of niacin metabolism in animals. Also, various N-oxides are known to be reduced by xanthine oxidase (86) and May (80) observed that in yeast, 16% of the added pyridine-N-oxide was accessible to bioreduction to pyridine.

How can one explain the ability of a wide variety of pyridine compounds in replacing nicotinic acid?. One possibility is that these analogs actually derepressed some systems in the staphylococci so that they now could synthesize niacin de novo, whereas niacin was a vitamin requirement before. If this is so, then a labelled analog should not be incorporated into NAD. When tritium labelled pyridine-N-oxide was added to the growth medium, about 1.8×10^{-3} counts were converted to 1 mole of NAD. Although this represents only 2% of the added counts, it should be noted that the labelled pyridine-N-oxide represents only 2.2×10^{-3}% of the total pyridine-N-oxide added to the growth medium. The radioactive NAD formed from pyridine-N-oxide reacted with yeast alcohol dehydrogenase, fluoresced after treatment with methylethylketone and ammonia,

and exhibited a maxima at 325 nm after treatment with 1 M potassium cynaide. All these properties agree with the characteristic of authentic NAD. Consequently, we can conclude that pyridine of pyridine-N-oxide itself was incorporated into NAD. There was no incorporation of radioactivity into NAD when both pyridine-N-oxide and NAD were incubated together in distilled water and the mixture chromatographed on paper. This suggested that the radioactive NAD formed, when washed-cell suspensions of staphylococci were incubated in the presence of labelled pyridine-N-oxide, was not due to the exchange reaction between the ^{3}H-pyridine-N-oxide and NAD. Similarly, when ^{14}C-quinolinic acid was incubated with NAD, there was no radioactivity on the NAD spot when the mixture was chromatographed on paper. Our data indicated that labelled quinolinic acid (6-^{14}C) was actually incorporated into the NAD molecule. At present, we do not know as to how quinolinic acid is converted into the formation of NAD in staphylococci.

To determine whether pyridine-N-oxide was first methylated and then further oxidized to produce niacin before the O group was removed, the staphylococcal cells were incubated with unlabelled pyridine-N-oxide, and either ^{14}C-methyl methionine or ^{14}C-formaldehyde. No radioactivity was found in the NAD synthesized when either compound was administered. Thus, the mechanism for the carboxylation of the pyridine ring

is completely unknown. Bioreduction of pyridine-N-oxide to pyridine may occur once the compound is within the cell. Although pyridine itself had no effect in stimulating staphylococcal growth, this may be due to the solvent-effect of free pyridine on the lipids of the cytoplasmic membrane. In sum, the stimulatory effect of the niacin analogs on staphylococcal growth could not be explained in terms of the formation of NAD analogs from the niacin analogs.

The major cause of the toxic effect of the niacin analogs in animal tissues is the formation of NAD analogs which inhibit or compete with NAD of certain dehydrogenases. Also the exchange reaction in animal tissues that is catalyzed by NADase, resulting in the formation of NAD analogs, is unlikely to occur in staphylococci since the staphylococcal NADase is normally associated with a heat-labile inhibitor. In addition, the activation of NADase by niacin analogs if it should occur would result in a reduction in the level of NAD, and this did not occur. Moreover, we could not substantiate Cote's (20) suggestion that the stimulatory effect of thionicotinamide on S. aureus growth was due to the breakdown of the niacin analog to yield niacin during autoclaving. We found that sterilization of the thionicotinamide either by membrane filtration or by autoclaving resulted in the same growth effect. Furthermore, we obtained no growth stimulation with addition of 0.5 µg/ml of

autoclaved thionicotinamide in the growth medium, the same concentration that Cote had reported to stimulate growth.

Most of the niacin analogs that were stimulatory to growth were required in 10- to 1000-fold higher concentrations than niacin. Of all the compounds tested, nicotinuric acid could replace niacin at the lowest concentration, even though it was still required at 10-fold higher level to achieve the same effect as either niacin and nicotinamide. With the analogs, a linear relation between cell, coenzyme content, and the analog concentration was more noticeable than with niacin. If it is assumed that the primary role of these analogs is the formation of NAD(P) coenzymes, then they probably must first be converted to niacin before they enter the niacin pathway to form coenzymes. Analysis of the staphylococcal cells grown in the presence of the analogs confirmed the fact that there was indeed a marked increase in NAD and NADP. Evidently, the niacin analogs are converted to niacin, or other intermediates and further synthesized into NAD(P) coenzymes which help to regulate the metabolic pathways.

Paralleled with the increased NAD(P) coenzymes in the presence of the analogs, there was also increased operation of the HMP pathway. One of these analogs, 3-acetylpyridine, in which the NH_2 group of the nicotinamide is replaced by a methyl

group (cf. Table 9), had been previously reported to be converted to niacin and to NAD in animal tissues (9,40). However, the NAD analog, acetylpyridine nucleotide (APAD) could be formed in some tissues. The formation of APAD only resulted in toxic effect in which certain dehydrogenases were inhibited by the APAD (57). Previous data on the effect of AP on microorganisms gave apparently rather conflicting results. For example, it was reported that AP added to the growth medium at 4 μg/ml could not stimulate the growth of Lactobacillus plantarum (131) whereas it stimulated the growth of Proteus species when added at much higher concentrations (Koser book). In our investigation, we observed that AP, when added to the media at relative high concentrations, could replace niacin as a growth factor for S. aureus, S. epidermidis, and several niacin-requiring strains of Gram-negative bacteria. Consequently, we have confirmed the results of AP with Proteus and suggest that one explanation for the lack of growth of L. plantarum with AP is due to the low concentration that was employed.

The purity of the niacin analogs was also checked to rule out the possibility that the growth stimulation by high concentrations of the analogs might actually due to contamination of the niacin analogs by niacin or nicotinamide. Two of the niacin analogs, AP and diethylnicotinamide, were tested

for purity by paper chromatography and the vitamins were observed by means of their reactions with cyanogen bromide and para-aminobenzoic acid, No detectable amount (less than 0.05 μg/ml) of niacin or niacinamide could be detected in 1 mg of AP. Since AP is volatile, and niacin and niacinamide are not, spotting 1 mg of AP on paper chromatogram was possible. The absence of niacin indicated that the AP contained less than 0.05 μg of niacin in 1 mg of the analog, and this is not enough to stimulate growth. Only one spot was found when 1 mg of diethylnicotinamide was spotted on paper. This was possible because the Rf value of the compound in the n-butanol-water solvent system was 0.65 as compared to the values of 0.30 and 0.75 for niacin and niacinamide. Even though the paper was overloaded with the diethylnicotinamide, the niacin or nicotinamide would have been visible.

Our results point to the difference in the degree of effectiveness of the niacin analogs as growth factors for staphylococci. It is possible that at low concentrations which did not stimulate growth, the analogs contributed to the supply of niacin requirement by being converted to NAD(P), but in amounts that were inadequate to meet the minimal growth requirement. When analogs that were required at relatively low concentrations, less than 100 μg/ml, the increased content of total NAD(P) was more evident than with compounds such as

AP, pyridine-N-oxide, or isoniazid, that were required in concentrations of 1 mg/ml. This suggest that the degree of conversion of the niacin analogs into niacin, and their subsequent incorporation into NAD(P) formation, could influence the rate of cell growth. In a different study, Imsande (52) showed that the incorporation of niacin into NAD paralleled cell growth. Our data suggest that staphylococci are able to perform a wide variety of reactions with pyridine compounds, previously unrecognized, leading to the formation of niacin. Although not examined in details, the results suggest that chemical properties of the analogs, related to the hindrance, electron withdrawal and/or increase in acidity, are involved in determining the relative effectiveness of the analogs.

SUMMARY

The levels of nicotinamide coenzymes and the free adenine nucleotides were examined after growth in several media, with or without the addition of niacin and/or thiamine, in order to determine whether the levels of these nucleotides were related to the changes in glucose catabolic pathways in staphylococci that occurred following these vitamin additions. The proportion of glucose metabolized via the hexosemonophosphate (HMP) pathway by resting staphylococcal suspensions was stimulated 3-fold when the cells were grown in a Vitamin-free Casitone growth medium to which niacin had been added in amounts >0.25 µg/ml. Concomitantly, there was a 10- to 15-fold increase in the NAD level, and a 2- to 3-fold increase in NADP. When 0.1 µg/ml of niacin was added, there was maximal growth but no stimulation of NAD synthesis or of HMP pathway activity. With the addition of higher niacin concentrations to resting niacin-deficient cells, metabolic changes similar to those in growing cells occurred. Even in the presence of protein inhibitors such as chloramphenicol, puromycin, or actinomycin D, the HMP pathway activity was increased, and the usual increases in NAD(P) were observed following the addition of niacin. Since the specific activities of the enzymes of the HMP pathway and of the other enzymes tested remained unchanged under conditions that precluded enzyme synthesis, any changes in enzyme levels observed in growing cells supplemented with niacin were

secondary to those of the nicotinamide coenzymes, and to the stimulation of glucose oxidation via the HMP pathway. In order to determine if NAD was effecting glucose oxidation via the HMP pathway, staphylococcal suspensions were incubated for 6 hr in buffered (pH 7.0)-glucose, under conditions that allowed the NAD to decrease and the NADP to remain constant. After 2 hr, when the initial NAD concentration (2.2 μmole/g. dry wt.) had decreased to 1 μmole, the percent of glucose oxidized via the HMP pathway dropped to 9%, reaching a value of 6% after 6 hr at which time the NAD had dropped to 0.5 μmole/g. The NADP concentration (0.45 μmole/g) did not decrease during the 6 hr period. Upon readdition of niacin to the buffered-glucose, the NAD content of these cells increased to 1.2 μmole/g after 2 hr additional incubation of the cells, and the HMP activity increased to 16%. NAD, apparently by increasing NADP turnover via the transhydrogenase system, is controlling the activity of this expandable HMP activity.

Following growth in unsupplemented Vitamin-free Casitone growth medium, staphylococci had ATP/AMP ratios close to 1. With the addition of niacin and thiamine to the growth medium, which stimulated the HMP and TCA cycle activities, the ATP/AMP ratios increased to values >5.

In the presence of thiamine, 22 of the 1-, 2-, 3-, or 4-substituted pyridine compounds could completely replace the

niacin requirement when they were added in either a synthetic medium, or in Vitamin-free Casitone; 3-substituted pyridine compounds such as 3-pyridylcarbinol and 3-acetylpyridine, were the most effective. Another 11 analogs yielded reduced rates of growth. There were 10 analogs, including 6-hydroxy-nicotinic acid, that were without effect, and 4, including 6-aminonicotinamide, that were inhibitory. Experiments using labelled quinolinic acid, isoniazid, and pyridine-N-oxide demonstrated that the analogs were converted to NAD(P) and not to the analogs of NAD(P). Other experiments with these labelled compounds showed that the requirement for high concentrations of analogs for growth was due to their limited uptake at low concentrations. Staphylococci were able to use many niacin analogs in concentrations greater than 1 mg/ml in the medium. Of the 40 compounds studied, precise structure to activity relationships were not evident, except that 3-substituted compounds were the most active.

LITERATURE CITED

1. Adams, H. 1965. Adenosine-5'-diphosphate and adenosine-5'-monophosphate, p. 573-580. In H. U. Bergmeyer, (ed.), Methods in enzymatic analysis. Academic Press Inc., New York.

2. Ahmad, F., and A. G. Moat. 1966. Nicotinic acid biosynthesis on prototrophs and tryptophan auxotrophs of Saccharomyces cerevisiae. J. Biol. Chem. 241:775-780

3. Anderson, B. M., C. J. Ciotti, and N. O. Kaplan. 1959. Chemical properties of 3-substituted pyridine diphosphopyridine nucleotides. J. Biol. Chem. 234:1219-1225

4. Atkinson, D. E., J. A. Hathaway, and E.S. Smith. 1965. Kinetics of regulatory enzymes: Kinetic order of the yeast diphosphopyridine nucleotide isocitric dehydrogenase and a model for the reaction. J. Biol. Chem. 240:2682-2693.

5. Atkinson, D. E. 1965. Biological feedback control at molecular level. Science 150:851-857.

6. Avigad, G. 1966. Inhibition of glucose 6-phosphate dehydrogenase by adenosine 5'-triphosphate. Proc. Natl Acad. Sci. 56:1543-1547.

7. Barker, K. L. 1967. Cofactor induced synthesis of D-glucose-6-phosphate: NADP oxidoreductase in the uterus. Endocrinology 81:791-797.

8. Beher, W. T., W. M. Holliday, and O. H. Gaebler. 1952. Studies of antimetabolites. II. 3-acetylpyridine. J. Biol. Chem. 198:574-578.

9. Beher, W. T., G. D. Baker, and M. Madoff. 1950. Studies of antimetabolites. V. Metabolism in vivo of 3-acetylpyridine. J. Biol. Chem. 234:2388-2390.

10. Behierkunst, A. 1966. Nicotinamide adenine dinucleotide in tubercle bacillus exposed to isoniazid. Science 152:525-526.

11. Berry, M. N. 1965. The effects of adenine nucleotides on pyruvate metabolism in rat liver. Biochem. J. 95:587-596.

12. Brin, M., S. S. Shohet, and C. S. Davidson. 1958. The effect of thiamine deficiency on the glucose oxidative pathway of rat erythrocytes. J. Biol. Chem. 230:319-326.

13. Brin, M., R. H. Yonemoto. 1958. Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. J. Biol. Chem. 230:307-317.

14. Burch, H. B., O. H. Lowry, and P. V. Dippe. 1963. The stability of triphosphopyridine nucleotide and its reduced form in rat liver. J. Biol. Chem. 238:2838-2842.

15. Caputto, R., H. S. Barra, and F. A. Cumar. 1967. Carbohydrate metabolism. Ann. Rev. Biochem. 36:211-243.

16. Chaykin, S. 1967. Nicotinamide coenzymes. Ann. Rev. Biochem. 36:149-170.

17. Cho, H. W., and R. G. Eagon. 1967. Factors affecting the pathways of glucose catabolism and the tricarboxylic acid cycle in Pseudomonas natriegens. J. Bacteriol. 93:866-873.

18. Cohen, G. N., and J. Monod. 1957. Bacterial permeases. Bacteriol. Rev. 21:169-194.

19. Collins, F. M., and J. Lascelles. 1962. The effect of growth conditions on oxidative and dehydrogenases activity in Staphylococcus aureus. J. Gen. Microbiol. 29:531-535.

20. Cote, L., and J. J. Oleson. 1951. Studies of nicotinamide derivatives. J. Bacteriol. 61:463-467.

21. Davidson, J. D., and P. Feigelson. 1957. Practical aspects of internal-sample liquid scintillation counting. Intern. J. Appl. Radiation Isotopes. 2:1-18.

22. Das, S. K., and G. C. Chatterjee. 1962. Pyrithiamine adaptation of Staphylococcus aureus. I. Adaptation and carbohydrate utilization. J. Bacteriol. 83:1251-1259.

23. Davis, P. D. 1961. The teleonomic significance of biosynthetic control mechanism. Cold Spring Harbour Sym. Bio. 26:1-10.

24. DeMoss, R. D., I. C. Gunsalus, and R. G. Bard. 1953. A glucose phosphate dehydrogenase in Leuconostoc mesenteriodes. J. Bacteriol. 66:10-16.

25. Dietrich, L. S., I. M. Friedland, and L. A. Kaplan. 1958. Pyridine nucleotide metabolism. Mechanism of action of the niacin antagonist, 6-aminonicotinamide. J. Biol. Chem. 233:965-968.

26. Dreyfuss, J., and A. B. Pardee. 1966. Regulation of sulfate transport in Salmonella typhimurium. J. Bacteriol 91:2275-2280.

27. Eagon, R. G. 1962. Pyridine nucleotide-linked reactions of Pseudomonas natriegens. J. Bacteriol. 84:819-821.

28. Eagon, R. G. 1963. Rate limiting effects of pyridine nucleotides on carbohydrate catabolic pathways of microorganisms. Biochem. Biophys. Res. Commun. 12:274-279.

29. Elek, S. D. 1959. Staphylococcus pyogenes and its relation to disease. E. and S. Livingstine Ltd., Edinburgh and London.

30. Ellinger, P., G. Fraenkel, and M. M. Abdel Kader. 1947. The utilization of nicotinamide derivatives and related compounds by mammals, insects and bacteria. Biochem. J. 41:559-568.

31. Feigelson, P., J. N. Williams, and C. A. Elvehjem. 1951. Inhibition of diphosphopyridine nucleotide requiring enzymes by nicotinamide. J. Biol. Chem. 189:361-369.

32. Ferguson, L. N. 1963. The modern structural theory of organic chemistry. Prentice Hall, Inc., Englewoods Cliffs, New Jersey.

33. Gershanovitch, V. N., N. A. Palkina, and G. L. Burd. 1963. The role of lactate in regulation of the enzymatic system synthesis participating in acetate oxidation in Staphylococcus aureus. Biochem. Biophys. Res. Commun. 13:12-19.

34. Glock, G. E., and P. McLean. 1955. Levels of oxidized and reduced diphosphopyridine nucleotides and triphosphopyridine nucleotide in animal tissues. Biochem. J. 61:388-390.

35. Goebell, H., and M. Klingenberg. 1963. DPN linked isocitrate dehydrogenase in near constant proportion to the respiratory chain. Biochem. Biophys. Res. Commun. 13:213-216.

36. Goldman, M., and H. J. Blumenthal. 1964. Pathways of glucose catabolism in Bacillus cereus. J. Bacteriol. 87:377-386.

37. Gray, C. T., J. W. T. Wimpenny, and M. R. Mossman. 1966. Regulation of metabolism in facultative bacteria. II. Effects of aerobiosis, anaerobiosis and nutrition on the formation of Krebs cycle enzymes in Escherichia coli. Biochim. Biophys. Acta 117:33-34.

38. Greengard, O., and M. Gordon. 1963. The cofactor-mediated regulation of apoenzyme levels in animal tissues. I. The pyridoxine induced rise of rat liver tyrosine transaminase level in vivo. J. Biol. Chem. 238:3708-3710.

39. Grenson, M.,M. Crabeel, J. M. Wiame, and J. Bechet. 1968. Inhibition of protein synthesis and stimulation of permease turnover in yeast. Biochem. Biophys. Res. Commun. 30:414-419.

40. Guggenheim, K., and E. J. Diamant. 1958. Effects of 3-acetylpyridine on metabolism of nicotinic acid in rats. Biochem. J. 69:56-59.

41. Hansen, R. G., and U. Henning. 1966. Regulation of pyruvate dehydrogenase activity in Escherichia coli K 12. Biochim. Biophys. Acta 122:355-358

42. Hanson, R. S., and D. P. Cox. 1967. Effect of different nutrition conditions on the synthesis of tricarboxylic acid enzymes. J. Bacteriol. 93:1777-1787.

43. Harary, I. 1957. Bacterial fermentation of nicotinic acid. I. End products. II. Anaerobic reversible hydroxylation of nicotinic acid to 6-hydroxynicotinic acid. J. Biol. Chem. 227:815-823, 823-831.

44. Herken, H., and K. Lange. 1969. Blocking of pentose phosphate pathway in brain of rats by 6-aminonicotinamide. Naunyn-Schiedebergs Arch. Pharmak. Exp. Path. 263: 496-499.

45. Hohorst, H. J. 1965. D-6-phosphogluconate, p. 143-145. In H. U. Bergmeyer, (ed.), Methods in enzymatic analysis. Academic Press Inc., New York.

46. Hoo, R., M. Wadke, J. M. Idriss, and H. J. Blumenthal. 1969. Levels of nicotinamide coenzymes and enzymes in metabolically altered Staphylococcus aureus. Bacteriol. Proc., p. 127.

47. Hughes, D. E. 1954. The inhibition of growth and cozymase synthesis in bacteria by halogen-substituted nicotinic acid. Biochem. J. 57:485-495.

48. Hughes, D. E. 1955. 6-Hydroxynicotinic acid as an intermediates in the oxidation of nicotinic acid by Pseudomonas fluorescens. Biochem. J. 60:303-310.

49. Iandolo, J. J., C. W. Clark, L. Bluhm, and Z. J. Ordal. 1965. Repression of Staphylococcus aureus in associative culture. Appl. Microbiol. 13:646-649.

50. Idriss, J. M., and H. J. Blumenthal. 1967. Effect of thiamine and niacin on the glucose catabolic pathways of staphylococci. Bacteriol. Proc., p. 127.

51. Iizuka, M., and D. Mizuno. 1967. Turnover of NAD in bacteria. Biochim. Biophys. Acta 148:320-327.

52. Imsande, J. 1961. Pathway of diphosphopyridine nucleotide biosynthesis in Escherichia coli. J. Biol. Chem. 236:1494-1497.

53. Imsande, J., and A. B. Pardee. 1962. Regulation of pyridine nucleotide biosynthesis in Escherichia coli. J. Biol. Chem. 237:1305-1307.

54. Johnson, W. J., and J. D. McColl. 1955. 6-Aminonicotinamide - a potent nicotinamide antagonist. Science 122:834.

55. Johnson, W. J., and J. D. McColl. 1956. Antimetabolite activity of 6-aminonicotinamide. Fed. Proc. 15:284.

56. Kaplan, N. O., A. Goldin, S. R. Humphreys, M. M. Ciotti, and J. M. Venditti. 1954. Significance of enzymatically catalyzed exchange reactions in chemotherapy. Science 120:437-410.

57. Kaplan, N. O., M. M. Ciotti, and F. E. Stolzenback. 1956. Reaction of pyridine nucleotide analogues with dehydrogenases. J. Biol. Chem. 221:833-843.

58. Kawasaki, T., I. Miyata, and Y. Nose. 1969. Thiamine uptake in Escherichia coli. II. The isolation and properties of a mutant of Escherichia coli defective in thiamine uptake. Arch. Biochem. Biophys. 131: 231-237.

59. Kilburn, J. O., K. D. Stottmier, and G. P. Kubica. 1968. Aspartic acid as a precursor for niacin synthesis by tubercle bacilli grown on 7H-10 agar medium. Am. J. Clin. Pathol. 50:582-596.

60. Kligler, I. J., and N. Grossowicz. 1941. The function of nicotinic acid in bacterial metabolism. J. Bacteriol. 42:173-192.

61. Kligler, I. J., N. Grossowicz, and S. Bergner. 1943. Studies on the role of niacin and thiamine in the metabolism of glucose by Staphylococcus aureus. J. Bacteriol. 46:399-403.

62. Klingenberg, M. 1965. Diphosphopyridine nucleotide (DPN), p. 528-530. In H. U. Bergmeyer, (ed.), Methods in enzymatic analysis. Academic Press Inc., New York.

63. Klingenberg, M. 1965. Triphosphopyridine nucleotide (TPN), p. 535-536. In H. U. Bergmeyer, (ed.), Methods in enzymatic analysis. Academic Press Inc., New York.

63. Knight, B. C. J. G. 1935. An essential growth factor for Staphylococcus aureus. Brit. J. Expt. Pathol. 16:315-326.

64. Knight, B. C. J. G. 1937. CXXVIII. The nutrition of Staphylococcus aureus. The activities of nicotinamide, aneurin (Vit. B_1) and related compounds. Biochem. J. 31:966-973.

65. Knowles, C. J., and L. Smith. 1970. Measurement of ATP levels of intact Azotobacter vinelandii under different conditions. Biochim. Biophys. Acta 197:152-160.

66. Kodicek, E., and K. K. Reddi. 1951. Paper chromatography of nicotinuric acid derivatives. Nature 168:475-476.

67. Kohler, E., H. J. Barrach, and D. Neubert. 1970. Inhibition of NADP dependent oxidoreductase by the 6-amino-nicotinamide analogue of NADP. Febs Letters 6:225-228.

68. Koser, S. A. 1968. Vitamin requirements of bacteria and yeast. C. C. Thomas, ILL.

69. Koser, S. A., and G. R. Baird. 1944. Bacterial destruction of nicotinic acid. J. Infect. Dis. 75:250-261.

70. Koser, S. A., and G. J. Kasai. 1947. The effect of large amounts of nicotinic acid and nicotinamide on bacterial growth. J. Bacteriol. 53:743-753.

71. Krebs, H. A., and J. M. Lowenstein. In Metabolic Pathways. (edited by D. M. Greenberg) Vol. 1, p. 129. Academic Press, N. Y., N. Y..

72. Lamprecht, W., and I. Trautscholf. 1965. Determination with hexosekinase and glucose-6-phosphate, p. 543-551. In H. U. Bergmeyer, (ed.), Methods in enzymatic analysis. Academic Press Inc., New York.

73. Lan, S. J., and L. M. Henderson. 1968. Uptake of nicotinic acid and nicotinamide by rat erythrocytes. J. Biol. Chem. 243:3388-3394.

74. Landy, M. 1938. Effect of pyridine compounds on the nutrition of Staphylococcus aureus. Nature 142:618.

75. Lessie, T., and F. C. Neidhardt. 1967. Adenosine triphosphate-linked control of Pseudomonas aeroginosa glucose-6-phosphate dehydrogenase. J. Bacteriol. 93:1337-1345.

76. Lichstein, H. C., and R. B. Ferguson. 1958. On the permeability of Lactobacillus arabinosus to biotin. J. Biol. Chem. 237:243-244.

77. London, J., and M. Knight. 1966. Concentrations of nicotinamide nucleotide coenzymes in micro-organisms. J. Gen. Microbiol. 44:241-254.

78. Luzzatto, L. 1967. Regulation of the activity of glucose-6-phosphate dehydrogenase by NADP+ and NADPH. Biochim. Biophys. Acta 146:18-25.

79. Magasanik, B. 1957. Nutrition of bacteria and fungi. Ann. Rev. Microbiol. 11:221-252.

80. May, A. 1957. Bioreduction of pyridine-N-oxide. Enzymologia 18:142-144.

81. McKee, R. W., A. Dickey, and M. E. Parks. 1968. NADase activity and glycolysis in Ehrlich-Lettre carcinoma and liver cell particulates. Arch. Biochem. Biophys. 126:760-763.

82. McIlwain, H. 1940. Pyridine-3-sulphonic acid and its amide as inhibitors of bacterial growth. Brit. J. Exptl Pathol. 21:136-147.

83. Model, P., and D. Rittenberg. 1967. Measurement of the activity of the hexose monophosphate pathway of glucose metabolism with the use of ^{18}O glucose. Variation in its activity in E. coli with growth conditions. Biochem. 6:69-79.

84. Montiel, F., and H. J. Blumenthal. 1965. Factors affecting the pathways of glucose catabolism and the tricarboxylic acid cycle in Staphylococcus aureus. Bacteriol. Proc., p. 77.

85. Morton, R. K. 1958. Enzymic synthesis of coenzyme I in relation to chemical control of cell growth. Nature 181:540-542.

86. Murray, K. N., and S. Chaykin. 1966. The enzymatic reduction of nicotinamide-N-oxide. J. Biol. Chem. 241:2029-2034.

87. Nakamura, S., S. Y. Nichizuka, and O. Hayaishi. 1964. Regulation of nicotinamide adenine dinucleotide biosynthesis by adenosine triphosphate. J. Biol. Chem. 239:PC 2717-2719.

88. Neuhoff, V. 1965. Verfahren zur quantitativen Extraktion der oxydierten und reduzierten Pyrinnucleotide aus Geweben. Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak. 250:302-306.

89. Neujahr, H. Y. 1966. Transport of B-vitamins in microorganisms. II. Factors affecting the uptake of labelled thiamine by non-proliferating cells of Lactobacillus fermenti. Acta Chemica Scand. 20:771-785.

90. Newell, P. C., and R. G. Tucker. 1966. The control mechanism of thiamine biosynthesis. Biochem. J. 100:517-524.

91. Newell, P. C., and R. G. Tucker. 1966. The derepression of thiamine biosynthesis by adenosine. Biochem. J. 100:512-516.

92. Nozawa, R., and D. Mizuno. 1969.. Replication and properties of DNA in nicotinamide adenine dinucleotide deficiency of Escherichia coli cells. Proc. Nat. Acad. Sci. 63: 904-910.

93. Ochiai, E. 1953. Recent Japanese work on the chemistry of pyridine-1-oxide and related compounds. J. Org. Chem. 18: 534-551.

94. Olivera, B. M., and I. R. Lehman. 1967. Diphosphopyridine nucleotide: A cofactor for the polynucleotide joining enzyme from Escherichia coli. Proc. Nat. Acad. Sci. 57: 1700-1704.

95. Ortega, M. V., and G. M. Brown. 1960. Precursors of nicotinic acid in Escherichia coli. J. Biol. Chem. 235: 2939-2945.

96. Osmond, C. B., and T. AP Rees. 1969. Control of the pentose phosphate pathway in yeast. Biochim. Biophys. Acta 184: 35-42.

97. Partridge, C. W. H., D. M. Bonner, and C. Yanoksky. 1952. A quantitative study of the relationship between tryptophan and niacin in Neurospora. J. Biol. Chem. 194: 269-278.

98. Pelczar, M. J., and J. R. Porter. 1940. The utilization of nicotinic acid and related pyridine compounds by the Proteus group of organisms. J. Bacteriol. 39: 429-434.

99. Pitsch, B. L., and M. Nakamura. 1963. Replacement of nicotinic acid requirement of Shigella sonnei by pyridine-3-sulfonic acid. J. Bacteriol. 86: 159-160.

100. Polakis, E. E., W. Bartley, and G. A. Meek. 1965. Changes in the activities of respiratory enzymes during aerobic growth of yeast in different carbon sources. Biochem. J. 97: 298-302.

101. Preiss, J., and P. Handler. 1958. Biosynthesis of diphosphopyridine nucleotide. I. Identification of intermediates. J. Biol. Chem. 233: 488-492.

102. Rogers, T. O., and H. C. Lichstein. 1969. Regulation of biotin transport in Saccharomyces cerevisiae. J. Bacteriol. 100: 565-572.

103. Rose, A. H., and W. J. Nickerson. 1956. Secretion of nicotinic acid by biotin-dependent yeast. J. Bacteriol. 72:324-328.

104. Sanwal, B. D. 1970. Allosteric controls of amphibolic pathways in bacteria. Bacteriol. Rev. 34:20-39.

105. Sauberlich, H. E. 1967. Biochemical alterations in thiamine deficiency- their interpretion. Am. J. Clin. Nutr. 20:528-542.

106. Saxton, R. E., V. Rocha, R. J. Rosser, A. J. Andreoli, M. Shimoyana, A. Kosaka, J. L. R. Chandler, and R. K. Gholson. 1968. A comparative study of the regulation of nicotinamide adenine dinucleotide biosynthesis. Biochim. Biophys. Acta. 156:77-84.

107. Schindler, J., and H. G. Schlegel. 1969. Regulation of the glucose-6-phosphate dehydrogenase of different bacterial species by ATP. Arch. Mikrobiol. 66:69-78.

108. Schmidt-Thome, J. 1948. The antibacterial action of 6-aminonicotinic acid. Z. Naturforsch. 38:136-137.

109. Scott, D. B., and S. S. Cohen. 1953. The oxidative pathway of carbohydrate metabolism in Escherichia coli. 2. Quantitative studies of glucose-6-phosphate and 6-phosphogluconate dehydrogenase. Biochem. J. 55:33-36.

110. Shuster, L., and A. Goldin. 1959. The effect of nicotinamide on incorporation in vivo of formate-C^{14}. J. Biol. Chem. 234:129-133.

111. Sie, H. G., and W. H. Fishman. 1967. Stimulation of pentose-phosphate pathway dehydrogenase enzyme activities in ethionine treated mice. Biochem. J. 106:769-775.

112. Smith, M. J. H., and J. A. Sturman. 1967. The mechanism of inhibition by salicylate of the pentose pathway in the human red cell. J. Pharm. Pharmac. 19:108-113.

113. Srere, K. 1967. Enzyme concentrations in tissues. Science 158:936-937.

114. Sriprakash, K. S., and T. Ramakrishan. 1969. Isoniazid and nicotinamide adenine dinucleotide synthesis in Mycobacterium tuberculosis. Indian J. Biochem. 6:49-50.

115. Strasters, K. C., and K. C. Winkler. 1963. Carbohydrate metabolism of Staphylococcus aureus. J. Gen. Microbiol. 33:213-229.

116. Streightoff, F. 1963. Inhibition of bacteria by 5-fluoronicotinic acid and other analogues of nicotinic acid. J. Bacteriol. 85:42-48.

117. Swartz, M. N., N. O. Kaplan, and M. E. Frech. 1956. Significance of "heat-activated" enzymes. Science 123:50-53.

118. Takebe, I., and K. Kitahara. 1963. Levels of nicotinamide coenzymes in lactic acid bacteria. J. Gen. Appl. Microbiol. 9 :31-40.

119. Telegdy-Kovats, M. 1967. Growth promoting effect of oxythiamine on Lactobacillus fermenti 37. Int. Z. Vitaminforsch. 37:329-330.

120. Theodore, T. S., and A. L. Schade. 1965. Carbohydrate metabolism of iron-rich and iron-poor Staphylococcus aureus. J. Gen. Microbiol. 40:385-395.

121. Tsuboi, K. K., J. F. Allan, and K. Fukunga. 1966. Cofactors and erythrocyte glycolytic capacity. J. Biol. Chem. 241:1616-1620.

122. Tsuboi, K. K. 1965. Limiting role of adenine nucleotide in the glycolysis of the human erythrocytes. J. Biol. Chem. 240:582-587.

123. Umbarger, H. E. 1960. End-product inhibition of the initial enzyme in a biosynthetic sequence as a mechanism of feedback control. Ann. Sym. Soc. Gen. Physiol. 7:67-68.

124. Wadke, M., R. Hoo, and H. J. Blumenthal. 1969. Effect of niacin replacement by its analogues on staphylococcal growth and carbohydrate metabolism. Bacteriol. Proc., p. 131.

125. Weitzman, P. D. J., and D. Jones. 1968. Regulation of citrate synthase and microbial taxonomy. Nature 219:270-272.

126. Wenner, L. E., J. H. Hackney, and F. Moliterno. 1958. The hexose monophosphate shunt in glucose catabolism in Ascites Tumor cells. Cancer Res. 18:1105-1114.

127. Wilson, A. C., and A. B. Pardee. 1962. Regulation of flavin synthesis by Escherichia coli. J. Gen. Microbiol. 28:283-303.

128. Winder, F. 1964. Early changes induced by isoniazid in the composition of Mycobacterium tuberculosis. Biochim. Biophys. Acta 82:210-212.

129. Witt, I., and I. Heilmeyer. 1966. Regulation of pyruvate decarboxylase synthesis by coenzyme. Biochem. Biophys. Res. Commun. 25:340-345.

130. Witt, I., and B. Neufang. 1970. Studies on the influence of thiamine on the synthesis of thiamine pyrophosphate-dependent enzymes in Saccharomyces cerevisiae. 215: 323-332.

131. Woolley, D. W., F. M. Strong, R. J. Madden, and C. A. Elvehjem. 1938. Antiblack tongue activity of various pyridine derivatives. J. Biol. Chem. 124:715-723.

132. Wright, J. A., and B. D. Sanwal. 1968. Regulatory mechanisms involving nicotinamide adenine nucleotides as allosteric effectors. II. Control of phospho-enolpyruvate carboxykinase. J. Biol. Chem. 244:1838-1845.

133. Zimmerman, S. B., J. W. Little, C. K. Oskinsky, and M. Gellert. 1967. Enzymic joining of DNA strands: A novel reaction of diphosphopyridine nucleotide. Proc. Nat. Acad. Sci. USA 57:1841-1867.

Approval Sheet

The dissertation submitted by Robert D. Y. Hoo has been read and approved by members of the Advisory Committee listed below.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

1 February 1971
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